THE NUTRITIVE VALUE OF THE PROTEIN IN TOBACCO-SEED OILMEAL [1]

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THREE FIGURES

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Mitchell, Hamilton and Beadles ('45) have pointed out that the various seed meals, as potential sources of protein for nutrition, are too often neglected in the quest for higher yields of oil. Vickery et al. ('32) investigating the nutritive properties of seed from Connecticut shade-grown tobacco found the whole ground seed to support good growth in albino rats and mice. Their chemical separation of various nitrogenous fractions proves the presence of certain amino acids. Since tobacco-seed oilmeal contains 36% crude protein on the oil-free basis, investigation of the nutritive value of this potential source of dietary protein appeared desirable.

A cold-pressed meal, containing about 30% crude protein and 21% oil, was used in this investigation to avoid any possible alteration of the proteins during evaporation of the oil solvent.

EXPERIMENTAL DATA

Growth promotion studies

For the growth studies weanling rats weighing 40–50 gm were transferred from the stock colony to group cages and fed ad libitum the respective experimental diets. With two

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exceptions each group of rats fed a given ration consisted of equal numbers of males and females.

The tobacco-seed oilmeal fed in both phases of the investigation was prepared by subjecting fine-ground seed to a pressure of 2500 lbs./sq. in. in a Carver press until no more oil was obtained. This fresh meal was incorporated immediately into rations or stored in the refrigerator until needed. Although the composition of various batches of pressed seed differed slightly, the following data, obtained upon the meal used for

TABLE 1

Composition of rations used in determining the nutritive value of tobacco-seed as a source of protein.

CONSTITUENT	RATION NUMBER									
	128	138	140	142	144	148	150	152	154	156
Tobacco-seed oilmeal [1]	60.0	33.3	33.3	33.3	33.3	33.3	33.3	33.3	33.3	50.0
Cornmeal		63.3			21.1					
Rolled oats			35.3		11.8					
Skimmilk powder				14.0	4.7					
Casein						5.0				
Lactalbumin							5.0			
Gelatin								5.0		
Wheat gluten									5.0	
Glucose	38.0		28.4	49.7	26.1	58.7	58.7	58.7	58.7	47.0
Salts [2]		2.4	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Sodium chloride	1.0									
Fortified corn oil [2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[1] Prepared by pressing ground seed in a Carver laboratory press.
[2] For composition, see Skinner and McHargue ('45).

determination of the biological value of protein are typical: crude protein (N × 5.34),[3] 25.8%; ether extract, 21.5%; crude fiber, 24.5%; moisture, 5.0%. Composition of rations is given in table 1. These were prepared fresh at least weekly.

Because sex played no significant role in rate of growth of rats fed the various rations, growth curves in figures 1–3 show the average weight of both sexes.

[3] Vickery et al. ('32) advocate the use of 5.34 instead of the usual 6.25 due to the high nitrogen content of the principal globulin of tobacco seed.

Three groups of rats were fed tobacco-seed oilmeal as the sole source of protein. Two of these groups received different lots of ration 128, one of which contained tobacco-seed oilmeal which had been autoclaved for an hour at a pressure of 15 lbs./sq. in. Both rations contained 18% protein. The third group was fed ration 156 containing 15% unheated protein. In figure 1 it will be seen that rats grew about ⅓ faster at the 18% level of intake than at the 15% level. The average increases in weight during the first 6 weeks were 32 and 23 gm, respectively. The poor growth of the remaining group, 12 gm in 6 weeks, suggests that the proteins of tobacco seed were damaged by the heat treatment. The curve shows that no improvement resulted from addition of 0.3% cystine to the ration.

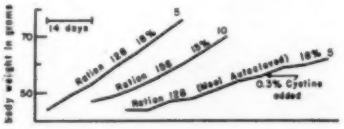

Fig. 1 Growth rate of animals with tobacco-seed oilmeal as the sole source of protein. First two curves show relationship of growth to level of protein intake. Third curve demonstrates detrimental effect of autoclaving the meal. Numerals above the curves indicate the number of animals fed each ration.

Since preliminary experiments demonstrated that proteins of tobacco-seed oilmeal alone support only slow growth, the next experiments were designed to ascertain the nature of the deficiency. Accordingly, four rations (148, 150, 152, and 154) were devised to demonstrate the supplementary value of casein, lactalbumin, gelatin and wheat gluten, respectively. They were incorporated at a level of 5% into rations containing sufficient tobacco-seed oilmeal to furnish 10% crude protein. It will be observed in figure 2 that fair growth was obtained on rations containing casein and lactalbumin supplements (rations 148 and 150, respectively). The response to supplementation with gelatin was less pronounced and was uninfluenced by addition of 0.5% cystine to the ration. Wheat gluten was even less satisfactory than gelatin as a supple-

ment for tobacco-seed protein. Of interest is the fact that during a period of 6 weeks the average gain of rats in the gluten group was 22 gm as compared with the gain, previously noted of 23 gm by rats consuming ration 156 in which 15% crude protein was furnished by tobacco-seed oilmeal alone. Since gluten is known to be deficient in lysine, it appeared probable that the proteins of tobacco-seed oilmeal were also deficient in this amino acid. That this is true was shown by the growth of six rats which for 4 weeks were fed ration 156 to which 0.5% of lysine monohydrochloride was added. During this period the average gain was 62 gm, exactly the increase in weight made by the rats fed the casein supplement (ration 148).

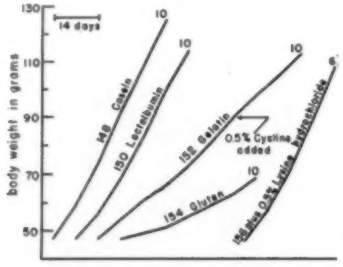

Fig. 2 Growth on tobacco-seed oilmeal with supplements of other proteins or lysine. First four curves represent animals fed tobacco-seed oilmeal at 10% protein level with 5% supplements of the proteins indicated. The last curve shows growth on tobacco-seed oilmeal at 15% protein level with addition of 0.5% lysine hydrochloride. Numerals above the curves indicate the number of animals fed each ration.

Use of a product such as tobacco-seed oilmeal as a major source of protein in rations can become practicable only if amino-acid deficiencies thereof are corrected by those materials which find frequent use in animal feeding. With this in mind, four additional rations (138, 140, 142, and 144) were fed to weanling rats for a period of 6 weeks. Each ration contained sufficient tobacco-seed oilmeal (33.3%) to furnish 10% crude protein. In three of them an additional 5% of protein was contributed by dried skimmilk, rolled oats, and

cornmeal, respectively. For the fourth ration (ration 144) these ingredients were added in such proportions that each contributed ⅓ of the supplementary protein. Since casein and lactalbumin when fed singly had proven to be effective supplements for tobacco-seed protein, it was expected that dried skimmilk would produce satisfactory response in growth. Upon examination of the growth curve for rats fed this supplement (fig. 3) it will be seen that the slope is almost the same as when casein was added at a level of 5% (fig. 2). Failure of cornmeal greatly to enhance growth on tobacco-seed protein

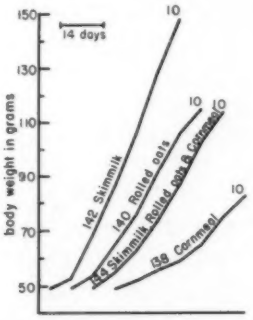

Fig. 3 Growth on tobacco-seed oilmeal with supplements of other protein-containing foods. All curves represent animals fed tobacco-seed oilmeal at 10% protein level with 5% protein supplements supplied by the foods indicated. In ration 144 each of the foods furnished ⅓ of the supplementary protein. Numerals above curves indicate the number of animals fed each ration.

was doubtless due to its low content of lysine. Rolled oats and the mixture of the three supplements were of approximately equal value in correcting the deficiencies of tobacco-seed protein.

Determination of digestibility and biological value

In order to ascertain the coefficients of digestibility and the relative biological values of the proteins of tobacco-seed oilmeal and milk, five pairs of young male rats were carried through the entire series of feeding periods, which lasted for

68 days. Each pair consisted of brothers of approximately the same initial weight. They were pair-fed (Mitchell and Beadles, '30) according to the reversal procedure of Mitchell et al. ('45) throughout the investigation.

The rats were housed in glass cages for collection of urine and feces. The cage proper consisted of a 1-gallon glass jug from which the bottom was removed by cutting off approximately 1 inch above the base. A tripod 5 inches in diameter and 9 inches high supported the inverted jug securely at a satisfactory height above the table.

The false bottom, cut from ½-inch mesh wire, was suspended by wires from the rim of the inverted jug; the depth to which it was lowered into the jug depended upon the size of the rat. A circular piece of wire gauze 3 inches in diameter was laid above the neck of the jug to retain the feces. In most instances the feces were deflected to the gauze thereby preventing to a great extent subsequent contamination with urine. A loop of fine wire attached rigidly to the wire gauze served as a handle by which it could be conveniently lifted from the cage so as to facilitate collection of the fecal pellets. The neck of the bottle was provided with a tightly fitting rubber stopper carrying a glass tube of such length that it extended about 2 inches into the 200-ml Erlenmeyer flask which served as a receptacle for the urine. A small quantity of 0.1 N sulfuric acid in the latter prevented growth of bacteria and loss of ammonia. The feces were removed daily, at which time residual urine was removed from the walls by washing with a stream of hot 0.1 N sulfuric acid until the total volume in the receiving flask was approximately 150 ml. During a given period, urinary samples for each animal were filtered through glass wool to remove particles of feed, combined in a 2½-liter bottle and preserved with thymol. Daily fecal samples likewise were combined and preserved under 95% alcohol containing a few drops of sulfuric acid. Collection periods, which were of 7 days' duration, were preceded by transition periods either 3 or 5 (usually 5) days in length. All nitrogen determinations on feces, urine and rations were made according

to the Gunning method in the Official and Tentative Methods of Analysis ('40) with the use of selenium as a catalyst as described by Lauro ('31).

Composition of the rations used in the determination of biological values is given in table 2. In order to eliminate variables among these rations (161A, 161B, 161C and 161D) sufficient tobacco-seed oil and Cellu Flour [3] were incorporated into 161B and 161C to raise the fat and fiber content to that of the two rations containing tobacco-seed oilmeal. All rations contained approximately 10% protein except the standardizing egg ration (Mitchell and Carman, '26) in which the concentration of protein was only 4%. Each ration was prepared in quantity sufficient for the entire metabolism period and stored in the refrigerator.

TABLE 2

Composition of rations used in the determination of biological values.

CONSTITUENT	RATION NUMBER			
	161A	161B	161C	161D
Tobacco-seed oilmeal [1]	33.1			
Skimmilk powder		29.8		
Dried egg [2]			5.4	
Glucose	63.0	38.8	61.1	
Salts [2]	2.6	1.6	3.7	
B vitamins [2]	0.3	0.3	0.3	
Fortified corn oil [3]	1.0	1.0	1.0	
Tobacco-seed oil [1]		7.1	7.1	
Cellu Flour		21.4	21.4	
Ration 161A				99.5
l(+)lysine monohydrochloride				0.5

[1] Prepared by pressing ground seed in a Carver laboratory press.

[2] For composition, see Skinner and McHargue ('45).

[3] Dried below 60°C. and extracted with ether.

Since it has been reported by Mitchell ('24 a) that the metabolic nitrogen and endogenous urinary nitrogen of a given rat change with time, each animal was placed on the

[3] A product obtained from the Chicago Dietetic Supply House, containing 37.8% crude fiber.

standardizing egg ration once near the beginning and once near the end of the experiment. In this way two values were obtained and plotted which permitted a specific value to be interpolated or extrapolated for each animal during the test period under observation.

From the nitrogen metabolism data taken the coefficients of true digestibility and biological values were computed in the usual way. The values thus obtained are reported for

TABLE 3

A comparison of the digestibility and biological value of the protein of tobacco-seed oilmeal with the protein of skimmilk powder.

RAT NO.	TOBACCO-SEED OILMEAL		TOBACCO-SEED OILMEAL PLUS LYSINE		SKIMMILK POWDER	
	True digestibility	Biological value	True digestibility	Biological value	True digestibility	Biological value
1	72 [1]	60 [1]			99	83
2	78 [1]	49 [1]			98	87
3	78 [1]	58 [1]			100	80
4	78 [1]	53 [1]			96	89
5	82 [1]	54 [1]			96	76
6	78	47	77	74	95 [1]	65 [1]
7	77	47	75	72	92 [1]	78 [1]
8	79	52	80	70	97 [1]	80 [1]
9	79	45	79	79	96 [1]	72 [1]
10	79	49	82	74	97 [1]	75 [1]
Avg.	78.0	51.4	78.6	73.8	96.6	78.5
Avg. 1–5	77.6	54.8			97.8	83.0
Avg. 6–10	78.4	48.0	78.6	73.8	95.4	74.0

[1] Average values of two or more test periods.

each animal in table 3. Averages of the individual values are recorded whenever an animal was fed a given ration during more than one period.

DISCUSSION OF RESULTS

Although a ration containing tobacco-seed oilmeal as the sole source of protein would be incomplete, the results indicate that this material may serve satisfactorily as a source of

dietary protein when fed in combination with other materials of moderately high lysine content. As Mitchell ('43) has pointed out, the value of a given protein as a supplement is probably more important than its biological value alone since few diets contain only one source of protein.

In the digestibility determinations the relatively high fiber content (8%) apparently had no effect upon the true digestibility since the values for skimmilk powder are in agreement with those reported by Mitchell ('24 b). This fact was also observed by Adolph and Wu ('34) using much higher concentrations of roughage. Results in table 3 show that rats 1–5 were, as a group, more efficient in utilization of protein consumed than their pair mates. The average biological value of tobacco-seed oilmeal protein when calculated from data obtained upon these rats was 54.8 as compared with 48.0 for rats 6–10. With skimmilk protein the biological values observed in the two groups as named were 83.0 and 74.0. Considering rats 6–10 since they only were fed the ration containing added lysine, it will be seen that this amino acid did not change the true digestibility but raised the biological value of the oilmeal protein from 48.0 to 73.8, approximately that found for skimmilk protein when fed to the same rats. These numerical values explain why the slopes of the growth curve for lysine supplemented ration (fig. 2) and that for the ration supplemented with skimmilk (fig. 3) are equal.

Mitchell and Carman ('24) have called attention to the superiority of "net protein" value to either digestibility or biological value alone as a basis for rating sources of dietary protein. Total protein content as well as the two other factors is involved in the calculation of this value. Pressed tobacco-seed oilmeal above gives a net protein value of 10.8% and with lysine 16.7%. Assuming no change in digestibility or biological value tobacco-seed oilmeal (extracted at less than 70°C.) gives a net protein value of 14.3%. Comparison with the net protein values of 21.3% for raw soy flour (Mitchell et al., '45), 10.6, 5.0, 3.5, and 6.0% for rolled oats, white flour, whole corn and navy beans, respectively, (Mitchell, '27) proves

the above forms of tobacco-seed oilmeal to rank high as a source of dietary protein.

The results of a previous investigation (Rapp et al., '46) show that although use of the cold-pressed oilmeal with its residual oil content was suitable for these experiments, the commercial storage problem created by the susceptibility of the oil to oxidation would very probably necessitate utilization of oil-free meal as a source of dietary protein.

SUMMARY

1. When tobacco-seed oilmeal was fed to rats at levels of 15 to 18% protein, the rats made only slow growth. The oilmeal was proved to be deficient in lysine.

2. When a ration containing tobacco-seed oilmeal as the source of protein was supplemented with casein, lactalbumin, skimmilk powder, or lysine, good growth was obtained. Rolled oats, cornmeal and gelatin were less satisfactory supplements to the oilmeal protein. Addition of wheat gluten was no more effective than increasing the proportion of tobacco-seed oilmeal so as to equalize the protein content of the two rations.

3. The biological values of the protein of tobacco-seed oilmeal and skimmilk powder when fed at a level of 10% were 51.4 and 78.5, respectively. Values for true digestibility of the two in the order named were 78.0 and 96.6. The biological value of the oilmeal protein when supplemented with lysine was approximately that of milk proteins.

4. Because of its relatively high net protein value tobacco-seed oilmeal deserves consideration as a source of dietary protein.

LITERATURE CITED

ADOLPH, W. H., AND MAO-YI WU 1934 The influence of roughage on protein digestibility. J. Nutrition, vol. 7, p. 381.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1940 Official and Tentative Methods of Analysis. 5th Ed., p. 26.

LAURO, M. F. 1931 Use of selenium as catalyst in determination of nitrogen by Kjeldahl method. Ind. Eng. Chem., Anal. Ed., vol. 3, p. 401.

MITCHELL, H. H. 1924 a A method of determining the biological value of protein. J. Biol. Chem., vol. 58, p. 873.

———— 1924 b The biological value of proteins at different levels of intake. J. Biol. Chem., vol. 58, p. 905.

———— 1927 The protein values of foods in nutrition. J. Home Econ., vol. 19, p. 122.

———— 1943 Biological methods of measuring the protein values of feeds. J. Animal Sci., vol. 2, p. 263.

MITCHELL, H. H., AND J. R. BEADLES 1930 The pair-feeding method in nutrition experiments and its application to the problem of cystine deficiencies in food proteins. J. Nutrition, vol. 2, p. 225.

MITCHELL, H. H., AND G. G. CARMAN 1924 The biological value for maintenance and growth of the proteins of whole wheat, eggs, and pork. J. Biol. Chem., vol. 60, p. 613.

———— 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., vol. 68, p. 183.

MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES 1945 The importance of commercial processing for the protein value of food products. I. Soybean, coconut and sunflower seed. J. Nutrition, vol. 29, p. 13.

RAPP, K. E., J. T. SKINNER AND J. S. MCHARGUE 1946 The nutritive value of tobacco-seed oil. J. Nutrition, vol. 31, p. 273.

SKINNER, J. T., AND J. S. MCHARGUE 1945 Response of rats to boron supplements when fed rations low in potassium. Am. J. Physiol., vol. 143, p. 385.

VICKERY, H. B., L. B. MENDEL, A. J. WAKEMAN, C. S. LEAVENWORTH AND F. A. MCCORMICK 1932 Chemical investigations of the tobacco plant. III. Tobacco seed. Conn. Agr. Expt. Sta. Bull. 339, p. 609.

THE NUTRITIVE VALUE OF TOBACCO-SEED OIL[1]

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TWO FIGURES

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Under present methods of producing tobacco in Kentucky, seed are used only for starting the following year's crop. However, with the increased practice of priming tobacco it is likely that a considerable quantity of seed could be produced without seriously affecting the quality of the leaf, provided a sufficient demand for the byproducts from the seed were created.

Mendel and Vickery ('32), investigating whole ground tobacco seed, obtained good growth with albino rats and mice even when the diet was 99% ground tobacco seed. Previous investigations at this Station by Scherffius and Woosley ('09) and McHargue et al. ('42) show that Kentucky tobacco seed contains about 40% of oil which has drying properties.

Although association of drying oils and nutrition is uncommon, Kaufmann ('41) reported that oil from the seed of certain European tobaccos can be used in foods. The purpose of this investigation was to ascertain the nutritive value of the oil from the seed of Nicotiana tabacum, which is commonly grown in Kentucky.

EXPERIMENTAL DATA

Before starting the feeding experiments, tobacco seed was analyzed for nicotine and nornicotine. Neither of these sub-

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

stances was found. This is in agreement with the results reported by Vickery and Pucher ('30) investigating the mature seed of Connecticut shade-grown tobacco. Il'in ('29) found that immature seed contained small amounts of the alkaloids and Mendel and Vickery ('32) reported the detection of nicotine in the sprouts and cotyledons after 9 to 11 days germination. However the latter investigators go on to show that this has little or no effect upon the nutritive properties of the seed.

The composition of each experimental ration used in this investigation is shown in table 1.

TABLE 1

Composition of rations (per cent).

CONSTITUENTS	RATION A	RATION B	RATION C	RATION D	RATION E
Fat or oil [1]	0	5	15	30	30
Casein [2]	18	19.1	21.4	24.7	24.7
Glucose	76.7	70.3	57.3	38.2	38.2
Salt mixture [3]	5.0	5.3	6.0	6.8	6.8
Vitamin supplements [4]	0.3	0.3	0.3	0.3	0.3

[1] Butterfat was obtained from melted creamery butter. The cotton-seed oil was a refined product sold for human consumption. Total tobacco-seed oil used in rations B–D was obtained by extraction with a low-boiling petroleum fraction, evaporation of the solvent and partial bleaching with fuller's earth. Ration E related only to pressed tobacco-seed oil which was untreated.

[2] Ratio of protein to energy intake remained constant throughout.

[3] Approximately that of Phillips and Hart ('35). Ratio of salts to energy intake remained constant throughout.

[4] Skinner and McHargue ('45) except that fat-soluble vitamins were dissolved in ether.

Digestibility determinations

Digestibility coefficients of tobacco-seed oil, cotton-seed oil and butterfat were determined with ad libitum feeding at three levels of fat intake on three groups of four young adult male rats, housed in individual wire cages. Each digestion period lasted 9 days and consisted of a 2-day transition period followed by 7 days during which food consumption was recorded and feces were collected daily, placed in bottles and kept in a refrigerator. During the initial digestion period each ani-

mal was fed fat-free ration A. In subsequent periods rations B, C and D (containing 5, 15 and 30% fat, respectively) were fed in the order named, one group of animals receiving tobacco-seed oil at the three levels, another cotton-seed oil and the third butterfat. All rations were kept in the refrigerator. At the end of a given 7-day collection period the pooled feces of each animal were dried at 100°C. and extracted with ether to determine the amount of fat excreted. The fat excreted on ration A was considered metabolic fat and the relationship between metabolic fat and fat-free food intake was assumed

TABLE 2

Coefficients of digestibility (per cent).

OIL OR FAT IN RATION	AT 5% LEVEL		AT 15% LEVEL		AT 30% LEVEL	
	Average	Range	Average	Range	Average	Range
Tobacco-seed oil	97.6	97.5–98.8	97.8	97.2–98.2	98.2	98.0–98.4
Cotton-seed oil	99.4	99.0–99.5	99.0	98.9–99.2	99.0	98.7–99.2
Butterfat	98.4	97.7–99.2	97.7	95.5–98.8	98.4	97.2–98.9

TABLE 3

Iodine number (Hanus).

OIL OR FAT IN RATION	INGESTED FAT OR OIL	FAT EXCRETED BY GROUP WHEN FED			
		No fat	5% fat	15% fat	30% fat
Tobacco-seed oil	148.3	51.6	47.4	42.8	44.5
Cotton-seed oil	103.1	54.4	60.7	56.1	56.9
Butterfat	30.2	44.4	36.6	38.8	38.5

to remain constant in subsequent periods as was done by Langworthy and Holmes ('15). The average coefficients of digestibility, as well as the ranges, for each fat intake level are given in table 2. Though cotton-seed oil showed a slight margin over the other two fats, there was very little difference between any of these fats when fed at the same level or the same fat when fed at different levels up to 30%.

The iodine number (Hanus method) was determined on each of the fats used in the rations and likewise on the composite samples of fecal fat excreted during the respective metabolism periods. Results are shown in table 3. Assuming the

composition of Kentucky tobacco-seed oil to be similar to that of the oil from European tobaccos analyzed by Lesyuis ('40) and Kaufmann ('41) and using the predicted digestibilities of Holt et al. ('35) the results in tables 2 and 3 were as anticipated.

Growth promotion studies

For the study of growth promotion, rations C and D of each fat were fed ad libitum to three matched groups of weanling rats (four male and four female) for a period of 4 weeks. Bulk rations were stored in closed jars at room temperature

TABLE 4

Growth data (ad libitum feeding for 4 weeks).

OIL OR FAT IN RATION STORED AT ROOM TEMPERATURE (DURING AUG., 1944)	TOTAL WEIGHTS OF GROUPS (GM.)					
	Group of 4 males			Group of 4 females		
	Initial	Final	Gain	Initial	Final	Gain
At 15% level						
Tobacco-seed oil	176	179	3	176	215	39
Cotton-seed oil	181	412	231	176	365	189
Butterfat	176	616	440	177	506	329
At 30% level						
Tobacco-seed oil	178	[1]	...	173	[2]	
Cotton-seed oil	177	706	529	173	499	326
Butterfat	178	687	509	172	533	361

[1] All animals in group lost weight and died. Average survival was 12 days.
[2] All animals in group lost weight and died. Average survival was 11 days.

(during August 1944). Results are given in table 4 as total-initial and -final weights for each group as well as total weight gained by the group. The animals receiving butterfat made the largest gains while those on tobacco-seed oil rations made the least gains. At the 15% level average weekly gains on tobacco-seed oil were almost negligible and at the 30% level all animals died before completion of the experiment as indicated by their average time of survival shown in table 4.

In view of the poor gains made by the animals on tobacco-seed oil rations, a duplication of this part of the experiment

was made using eight additional animals at the 15% level and six animals at the 30% level. The previous results were confirmed and every animal which survived the entire experiment showed erratic weight changes.

It was observed in the foregoing experiments that after storage for a short time at room temperature the tobacco-seed oil rations became spongy and had a pronounced odor even though the oil used in making the rations had been stored in closed bottles at room temperature for several months with no evidence of becoming rancid. This indicated that the rate of oxidation of the oil was increased quite rapidly by the higher temperatures and greatly enlarged surface area. Since the latter could not be reduced materially, control of temperature provided the only convenient means of retarding oxidation.

Preliminary tests showed that about half of the oil contained in seed could be expressed in a relatively short time by a Carver laboratory press at maximum pressure. The oil obtained in this way possessed the same physical and chemical constants as the bleached extracted oil. Substitution of this oil in subsequent experiments made possible a supply of freshly prepared oil and also eliminated the heat treatment necessary to remove the solvent from the extracted oil.

To determine the extent to which the nutritive value of the ration was affected by development of rancidity, two groups of six young rats each were fed ration E. One group was fed from bulk ration stored at room temperature while the ration for the other group was refrigerated. The rats were fed daily from the respective stocks and any unconsumed ration from the day before was discarded. Individual growth curves for these animals over a period of 5 weeks are shown in figure 1. Attention is called to the second break in the curves at the right demonstrating a pronounced response to freshly mixed ration. Also it might be mentioned that animals 1 and 4 in the curves at the right were started together on fresh ration, animals 2 and 5 were started 3 days later being fed from the same bulk ration and 3 and 6 were started 6 days still later, on the

ration corresponding to that of the second week of the first pair. The growth curves of the pairs, showing the extremes in growth as related to age of the ration, are similar.

In their review Burr and Barnes ('43) point out that rancid fats catalyze the destruction of certain fat-soluble vitamins. In view of the previous experiments it seemed necessary to determine whether the poor growth of animals on tobacco-seed oil ration was due primarily to resultant vitamin deficiencies.

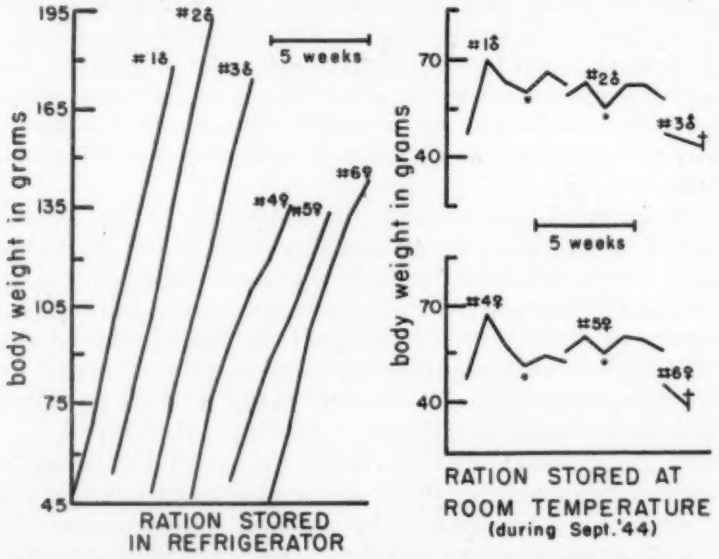

Fig. 1 Individual growth curves of animals on 30% pressed tobacco-seed oil ration. * Freshly mixed ration fed at this point. † no. 3 ♂ died in 14 days, no. 6 ♀ died in 9 days.

Consequently, seven young animals (ca. 3 weeks old) were put on total oil ration D stored at room temperature. In addition, two of the animals received a daily supplement of fat-soluble vitamins, two other animals received B vitamins and three animals were fed both fat-soluble and B vitamins. These daily supplements were given individually in separate containers and in amounts equivalent to those furnished by 10 gm

of freshly prepared ration. All animals consistently lost weight and survived for periods varying from 4 to 28 days.

Comparison of the average growth rates of animals on butterfat ration D and refrigerated tobacco-seed oil ration E, 31.8 and 26.2 gm/week, respectively, for males and 22.6 and 17.9 gm/week, respectively, for females, indicates better growth on butterfat with ad libitum feeding. However, it was observed that the animals of the former group consumed more ration than those on the tobacco-seed oil ration. Consequently, twelve rats were paired as closely as possible with regard to initial weight, sex and litters and fed controlled

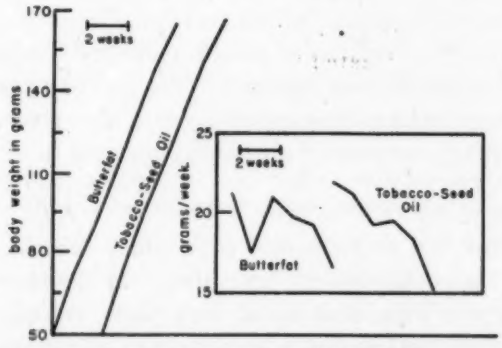

Fig. 2 Average growth of six animals on 30% ration stored in refrigerator (paired-feeding). Insert shows average weekly gains.

amounts of butterfat ration D and ration E (refrigerated) for a period of 6 weeks. During the first 2 weeks of the experiment the animals on tobacco-seed oil established a lead in total weight gained, part of which was maintained for the entire 6 weeks. Over the whole period differences in weight gains of the individual pairs were insignificant except in one instance. The food intake of this pair was definitely limited by the animal on the butterfat ration. Average cumulative growth and weekly gain curves for the six pairs are shown in figure 2 and insert, respectively.

Effect on functional activity

In view of the possible destruction of certain of the fat-soluble vitamins in the tobacco-seed oil rations, the animals surviving the experiment on refrigerated and non-refrigerated tobacco-seed oil rations (fig. 1) were put on stock ration in mated pairs designed to determine if reproduction had been permanently impaired in either the males or females by either of the experimental diets. Those animals which had received the unrefrigerated ration and were most likely to be sterile were paired with animals of known fertility from the stock colony. At the time of taking the animals off the experimental diets they had not reached maturity with respect to growth, those on non-refrigerated ration averaging only 55 gm. Since sexual maturity is related to growth maturity a delay in production of offspring was expected. This proved to be the case with all the animals and especially so with the extremely small ones. However, eventually all animals proved to be fertile.

DISCUSSION OF RESULTS

Results of the experiments show that, for all practical purposes, the coefficients of digestibility of the three fats investigated are high and equal and, under certain feeding conditions, the fats support good growth in young animals. The observation that growth of pair-fed rats was the same on rations containing butterfat and tobacco-seed oil, respectively, is in keeping with the comparative nutritive values of butterfat and other vegetable oils reported by Deuel et al. ('43). It is doubtful if flavor contributed to the superiority of butterfat under ad libitum feeding since unrefined tobacco-seed oil possesses no particularly objectionable flavor. Moreover, Boutwell et al. ('44) found that removal of flavoring agents from butterfat or addition of diacetyl to corn oil had little effect upon the comparative nutritive values of these two fats.

Temperature and exposed surface area have a pronounced effect on the rate of oxidation of tobacco-seed oil and in this

respect it follows the pattern of other drying oils. However, if certain precautions are taken when the oil is most subject to oxidation (i.e., after mixing with other foods so that a large area is exposed) there is little reason for it being unsuited for dietary purposes. Even these precautions are unnecessary as long as the oil is isolated and stored in closed containers. Although this property is less pronounced with the commonly accepted edible fats and oils it is well recognized that refrigeration is essential when these are incorporated in food mixtures.

The poor performance of the animals on unrefrigerated rations was not due primarily to destruction of vitamins present as the vitamin supplement experiment showed. Also no permanent ill effects were noted which might indicate severe vitamin deficiencies when proper precaution was taken.

SUMMARY

1. When tobacco-seed oil was fed to rats at levels of 5, 15 and 30% of the respective rations it gave an average coefficient of digestibility of 97.9 as compared with 99.1 and 98.2 for cotton-seed oil and butterfat, respectively.

2. With paired feeding of refrigerated rations containing 30% of tobacco-seed oil and butterfat, respectively, growth rates of rats did not differ significantly. When fed ad libitum, the difference in consumption of the two rations produced a greater rate of growth in rats fed butterfat.

3. Of six rats fed refrigerated ration and four animals fed non-refrigerated ration containing 30% tobacco-seed oil for 5 weeks, during the period when growth is usually maximum, all proved to be fertile upon reaching maturity.

4. Both temperature and surface area affected the rate of oxidation of the oil. When surface area was small, as in the case of the isolated oil, no particular care in storing was necessary; but when the oil was incorporated in a ration and kept for any length of time, refrigeration was necessary.

LITERATURE CITED

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1944 Effect of flavor on nutritive value of fats. Proc. Soc. Exp. Biol. Med., vol. 55, p. 153.

BURR, G. O., AND R. H. BARNES 1943 Non-caloric functions of dietary fats. Physiol. Rev., vol. 23, p. 256.

DEUEL, H. J., JR., E. MOVITT AND L. F. HALLMAN 1943 Comparative nutritive value of butter and some vegetable fats. Science, vol. 98, p. 139.

HOLT, L. E., JR., H. C. TIDWELL, C. M. KIRK, D. M. CROSS AND S. NEALE 1935 Studies in fat metabolism. I. Fat absorption in normal infants. J. Pediat., vol. 6, p. 427.

IL'IN, G. S. 1929 Nicotine content of maturing tobacco seed. U.S.S.R. State Inst. Tobacco Invest. Bull. 57 (in Russian). Biol. Abstracts, vol. 6, p. 1894.

KAUFMANN, H. P. 1941 Bulgarian tobacco-seed oil. Fette u. Seifen, vol. 48, p. 193. Chem. Abstracts, vol. 36, p. 3691.

LANGWORTHY, C. F., AND A. D. HOLMES 1915 Digestibility of some animal fats. U. S. Dept. Agr. Bull. 310, 23 pp.

LESYUIS, A. 1940 Tobacco-seed oil. Masloboino Zhirovoe Delo, vol. 16, p. 12. Chem. Abstracts, vol. 34, p. 7130.

MCHARGUE, J. S., C. W. WOODMANSEE AND K. E. RAPP 1942 New uses for low-grade tobacco. Ky. Agr. Exp. Sta. Bull. 439, 12 pp.

MENDEL, L. B., AND H. B. VICKERY 1932 Chemical investigations of the tobacco plant. III. Tobacco seed. Part I. The nutritive properties of tobacco seed. Conn. Agr. Exp. Sta. Bull. 339, p. 609.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., vol. 109, p. 657.

SCHERFFIUS, W. H., AND H. WOOSLEY 1909 Tobacco. Ky. Agr. Exp. Sta. Bull. 139, 36 pp.

SKINNER, J. T., AND J. S. MCHARGUE 1945 Response of rats to boron supplements when fed rations low in potassium. Am. J. Physiol., vol. 143, p. 385.

VICKERY, H. B., AND G. W. PUCHER 1930 Chemical investigations of tobacco. Conn. Agr. Exp. Sta. Bull. 311, p. 234.

MENTAL RESPONSE TO ADDED THIAMINE

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SIX FIGURES

(Received for publication May 31, 1945)

The present paper gives a short[1] account for the nutritionist of an experimental study of psychological response to liberal thiamine intake in children of school age. The study employed all the suitable children of the 120 living in the Presbyterian Orphans' Home at Lynchburg, Virginia. Without their knowledge, they were assigned to two groups matched very closely in pairs as to age, sex, stature, mental ability and educational achievement. All the children subsisted at the same table without segregation but the children of one group received daily a tablet containing 2 mg of thiamine while those of the other group received daily an indistinguishable placebo.[2] Measurements were made at regular intervals of the progress of the children in mental and physical skills by a systematic program of tests.

GENERAL PROGRAM OF THE STUDY

The study covered three periods: (1) Six weeks' trial of thiamine supplement utilizing a wide range of test tasks, May 15–July 1, 1941 (Harrell, '43). (2) First year-long experiment with selected tasks, September 1942–September

[1] To be published in full under the title "Further Effects of Added Thiamin on Learning and other Processes," Bureau of Publications, Teachers College, Columbia University, New York.

[2] Tablets donated by Hoffmann-La Roche, Incorporated.

1943. (3) Second year-long experiment involving interchange of some children between groups, September 1943–September 1944. Child personnel in the orphanage gradually changed during the course of the experiments, thus somewhat limiting the number of matched pairs for which comparisons could be made during the longer periods. All the children participated in the program insofar as they were able and their scores were recorded to aid in maintaining the morale of the experiment. However, certain of the tests require ability to read. Full comparisons of performance are therefore largely limited to the age range 9 to 19 years. The actual number of pairs participating in all tests at each interval is indicated in tables 2 and 3. It varied from twenty to fifty-five pairs.

Orphanage regimen and dietary

The orphanage is located on a 200-acre farm on which the children live, work and attend school. Food is prepared for all the children in a single kitchen and served in a single dining room at eighteen tables, each accomodating one supervisory adult and seven children. For economy the proportion of cereals in the dietary is high and that of meat is low. A gallon of excellent quality milk from a pure-bred Jersey herd which is tended by the older boys of the Home, is, however, served at each table at every meal. Butter and cheese from the farm dairy are liberally available. Menus are, however, repetitive offering little variety; those of October 18, 19, 20 and 21, 1942, are listed in table 1 as typical.

Thiamine in the diet

Dietary surveys by nutritionists [3] of Columbia University were made during several days in June 1941 and again in October 1942. Weights of all foods eaten in the orphanage on the survey days were taken and the thiamine intake per child was calculated from tables (Taylor, '42) as 0.9 and 1.0 mg, respectively, per day during the two periods. No deduction

[3] Dr. Mary Robertson and Miss Eunice Peterson.

TABLE 1

Illustrative menus of the orphanage. (October 18, 19, 20 and 21, 1942.)

DAY	BREAKFAST	DINNER	SUPPER
Sunday	Corn flakes; biscuits, baking powder, white flour (2/person); apple sauce; butter; milk	Ham, cold, boiled (1 slice per person); green beans, canned; potato salad; bread, whole wheat (2 slices per person); pineapple cake; milk	Peanut butter sandwich, white bread (1/person); cherry preserves sandwich, white bread (1/person); cookie (1/person); milk
Monday	Oatmeal; biscuits, baking powder, white flour (2/person); butter, milk	Vegetable soup; biscuits, baking powder, white flour (2/person); bread pudding; milk	Pork and beans, canned; cheese, 1 oz./person; biscuits, baking powder, white flour (2/person); milk
Tuesday	Oatmeal; molasses, canned; biscuits, baking powder, white flour (2/person); butter; milk	Dried beans, stewed; turnip greens; corn bread, white meal (1 square/person); milk	Spaghetti; salmon, cold, canned; biscuits, baking powder, white flour (2/person); apple sauce, milk
Wednesday	Corn flakes; biscuits, baking powder, white flour (2/person); butter; milk	Green beans, canned; mashed potatoes; tomatoes, stewed; corn bread, white meal (1 square/person); milk	Rice, white, boiled; carrot strips, raw; biscuits, baking powder, white flour (2/person); milk

was made for possible excessive cooking losses. During 1943–1944, in spite of precautions, enriched flour was encountered on two occasions and may have been used for a considerable period.

Parity of groups

Immediately prior to each experimental period, data were taken for each individual child including height, weight, age, sex, educational achievement, length of residence in the institution and his scores in two intelligence tests, namely, the I.E.R. Intelligence Scale CAVD and either the Otis Tests of Mental Ability (first period) or the Kuhlmann-Anderson Intelligence Tests (second and third periods).

On the basis of these data, the children were assigned to two well-matched groups by Dr. Ella Woodyard and Dr. Grace MacLeod, psychologist and nutritionist, respectively, of Teachers College, Columbia University, neither of whom participated in the subsequent observations of the children. Choice of the group to serve as control and that for experiment was also made by these individuals and was kept secret from the children and from all adults who participated in measurements of the children. Possibility of subjective bias was thereby avoided.

Assignment of children was primarily such as to match closely the mental abilities in each group, child for child. It was also possible to adjust assignments to secure comparability in age, size, sex, educational status and ratio of weight to expected weight. Eighty per cent of the children in this orphanage have one or more siblings there, so it was often possible to secure representation in each group of identical parentage and, accordingly, similarity of early home life and economic background. The groups as finally constituted were so similar that no factor known in advance offered a basis for anticipating differences in achievement.

Supplementation procedures

After secret assignment of the children and secret choice of the experimental group, Drs. Woodyard and MacLeod

placed in a series of envelopes, each bearing the name of a child, a suitable number of tablets of the appropriate sort whether a placebo or an indistinguishable tablet containing 2 mg of thiamine. These envelopes were dispatched to the orphanage where the matron of each dormitory cottage took custody of the appropriate ones and administered a tablet daily, usually at bed time, to each child from the envelope marked with his name. The supply of tablets was replenished as required in the same manner so that no participant in the study, either child or adult, could distinguish the experimental subjects from the control subjects, or could say, for example, whether Tom and Dick belonged to the same group or to different groups.

Measurements of performance

In the 6 weeks' trial from May 15 to July 1, 1941, the tasks were numerous and varied and were performed at frequent intervals, the children working at them daily. During the two experiments of year-long duration, the number of tasks was reduced to those which previous experience had indicated were most satisfactory and informative. All tasks were, however, such as are recognized and used currently by psychologists. In all cases, tasks were selected to be well within the range of the children's abilities, to engage and hold their interest and to be capable of objective measurement. Selection of the tasks to be used was made with the advice of Dr. Arthur I. Gates, Dr. Helen M. Walker, Dr. Robert L. Thorndike and Dr. Ella Woodyard, all of the Psychology Department of Teachers College, Columbia University.

Tests of the selected activities were administered by persons of professional experience in their use. The intelligence tests were administered by Dr. Woodyard, Dr. Cora Friedline of Randolph-Macon Woman's College and Miss Loulie Burner of Asheville College. Dr. Lyman Abbott of the Wilmer Ophthalmological Institute of the Johns Hopkins Hospital and University, assisted by Miss Lelia Robertson, tested the children in visual efficiency with the Snellen chart. The tests

of binocular vision [4] were made by Misses Todd and Dameron of the Lynchburg public schools. The measures of height and weight and the incidence of colds among the children were recorded by the principal and the assistant principal of the orphanage school.

During the 6 weeks' trial the numerous and varied tasks were repeated nine times by each child. These tasks are indicated at the bottom of figures 1 and 2. The nature of most of the tasks will be evident but a few require description. "Problems" signifies Monroe's Standardized Reasoning Tests in Arithmetic. "Mixed Fundamentals (Thorndike)" involve addition and subtraction; those of Woody-McCall involve addition, subtraction, multiplication and division. "Sums of Ten" consist of encircling two adjacent numbers whose sum is ten on a page of printed numbers. "Completion" signifies completion of fifteen simple geometric designs. "Number Span" consists of reproducing in a designated space on the obverse side of the paper series of numbers printed on the face. "Code Learning" or "Code Substitution" refers to the writing of the proper associated digit beneath each of five geometric symbols recurring repeatedly but in random order on a page of printed symbols. "Right hand" and "left hand" refer to strength of grip as measured by a dynamometer. Time limits were progressively shortened for successive trials of the timed tasks except for Code Substitution which was invariably practiced 5 minutes each time throughout all experiments.

A modified program of tests was adopted for the longer term experiments, as required by considerations of expense, availability of expert personnel and the morale of the children during prolonged repetitive drill. The schedule of tests was that indicated at the bottom of figure 3.

Some explanations of these choices are required. The Kuhlmann-Anderson Intelligence tests were substituted for the Otis Intelligence tests used in the earlier period because

[4] Keystone Telebinocular was loaned by the public school system of Lynchburg, Virginia.

they were better adapted to the younger children who were included in the longer experiments. Due to the withdrawals of children from the Home as they reached the age of self-support, a process which was much hastened by war conditions including the draft for military service, the average age of

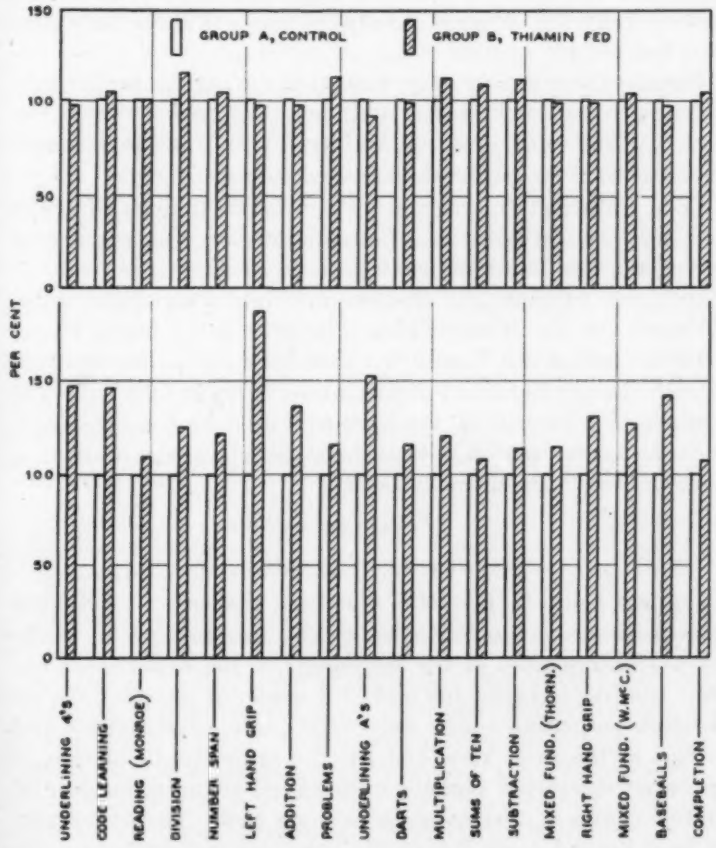

Fig. 1 (Upper graph) Mean standing of groups A and B at beginning of experiment (letting mean initial score of group A be 100%).

Fig. 2 (Lower graph) Relative mean gains made by groups A and B in 6 weeks of practice (letting gain of group A be 100%).

the available subjects was approximately 2 years younger during the later longer term experiments. Withdrawals progressively reduced the number of pair comparisons which could be made at successive intervals.

Visual perception tests were emphasized in the long experiments for the sake of including objective measurement of a sensory function. Expert assistance was available for visual tests but not for auditory.

Reaction time could not be measured during the earlier trial period for lack of suitable equipment. It was, however, included in the later program because of the high significance attached to it by professional psychologists.

Code Substitution was one of the original tests of which the children did not tire. Consequently it was carried on during the two later full year periods as well.

Incidence of colds and changes in height and weight would obviously be highly unreliable measures when taken over a 6 weeks' period but it was felt that they should be observed over the longer period of confirmatory experiments. Likewise changes in Educational Achievement could be measured only over the longer period, a consideration which also applies in some degree to the memory tests.

RESULTS

Six weeks' trial

Figures 1 and 2 (Harrell, '43) show, respectively, the comparative average performance of the two groups of thirty-seven children each at the beginning of the experiment and their relative gains at the end of 6 weeks of practice. In the eighteen test tasks which were repeatedly practiced by both groups at the same time and for the same length of time, it appeared significant that the vitamin fed group surpassed the control group in average gain in every task. The superiority of gain ranged from 7% to 87% and averaged 27% for the program of eighteen practiced tasks as a whole.

Statistically there were large individual variations of gain within each group. The significance of the mean difference

was computed for each task in the program after first finding the difference in gain separately for each of the thirty-seven pairs of children who performed all the tasks. The t-ratio which is the ratio of the mean to its standard error, (Student, '25) is given in table 2 for the differences in gain exhibited by the pairs of children in each of the eighteen activities. For seven of the tasks the significance is high; for eleven tasks the

TABLE 2

Superiority of thiamine fed group B over control group A in gain during 6 weeks' trial period, May–July, 1941. (37 pairs of children.)

ACTIVITY	T-RATIO	PROBABILITY THAT SUPERIOR GAIN WAS DUE TO CHANCE
1. Underlining 4's	3.33	0.002
2. Code learning	3.13	0.004
3. Reading (Monroe)	2.17	0.04
4. Division	2.11	0.04
5. Number span	2.09	0.04
6. Left-hand grip	2.07	0.04
7. Addition	2.07	0.04
8. Problems in arith.	2.00	0.05
9. Underlining A's	1.33	0.2
10. Darts	1.28	0.2
11. Multiplication	1.07	0.3
12. Sums of ten	1.03	0.3
13. Subtraction	0.67	0.5
14. Mixed fund. (Thorndike)	0.65	0.5
15. Right-hand grip	0.63	0.5
16. Mixed fund. (Woody-McCall)	0.61	0.5
17. Baseballs	0.60	0.5
18. Completion	0.54	0.6

t-ratios are small enough to permit the assumption that the measured superiorities may have been due to chance. However, it is almost inconceivable that by chance alone all eighteen superiorities should be in the same direction.

First year-long experiment, 1942–1943

Prior to the beginning of this experiment the groups were re-equalized by Drs. MacLeod and Woodyard as previously described in order to include newcomers to the orphanage in

the long term observations. Figure 3 [5] shows in graphic form the comparative gains in proficiency made by the two groups of fifty-five children each in the selected activities during the

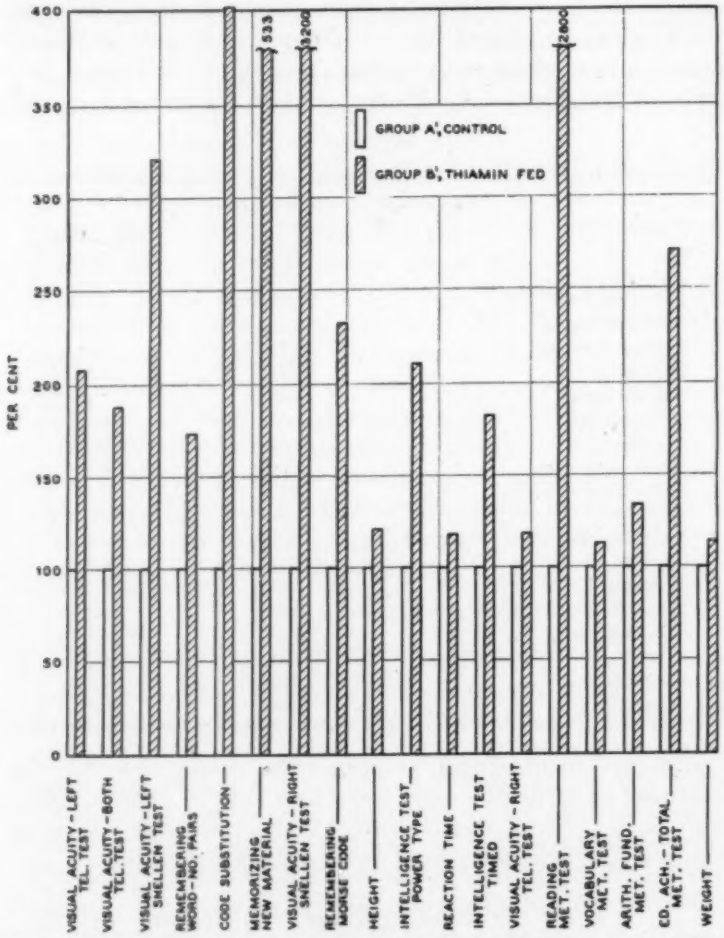

Fig. 3 Relative mean gains made by groups A′ and B′ in a year, 1942–1943 (letting gain of group A′ be 100%).

[5] Tabular data from which figure 3 was drawn are given in the full account, "Further Effects of Added Thiamin on Learning and other Processes," Bureau of Publications, Teachers College, Columbia University, New York.

year. In all fifteen tests the vitamin fed group surpassed the control group in average gain. The t-ratios, one for each activity, were computed as before and are given in table 3. Eight of them are so large that assumption of chance is difficult to justify, especially in view of the fact that all the superiorities are in the same direction.

TABLE 3

Superiority of thiamine fed group B' over control group A' in gain in first year-long experiment, September 1942–September 1943.

ACTIVITY	NO. OF PAIRS	T-RATIO	PROBABILITY THAT SUPERIOR GAIN WAS DUE TO CHANCE
1. Visual acuity, left eye telebinocular test	40	4.91	.000002
2. Visual acuity, both eyes telebinocular test	40	3.84	.0001
3. Visual acuity, left eye Snellen test	45	3.41	.002
4. Remembering: word-number pairs	20	3.23	.002
5. Code substitution	51	3.20	.003
6. Memorizing new material	47	2.83	.007
7. Visual acuity, right eye Snellen test	45	2.75	.01
8. Remembering: Morse code	20	2.70	.02
9. Height	43	2.28	.03
10. Intelligence power type test	55	2.15	.04
11. Reaction time [1]	52	2.04	.05
12. Intelligence timed test	48	1.85	.07
13. Visual acuity, right eye telebinocular test	40	1.69	.1
14. Educational achievement Metropolitan test, total score	39	1.62	.1
15. Weight	43	.94	.3

[1] Choice Reaction Time device was loaned by the Traffic Engineering and Safety Department, American Automobile Association, Washington, D. C.

Second year-long experiment, 1943–1944

With slight changes in the program of tests, the experiment was carried on through a second year after regrouping half the number of children so that of thirty (out of the first year's total of sixty) pairs, the member who had received placebos the first year received thiamine tablets the second year, and the member who had received thiamine the first year received placebos the second year. The purpose of this reversal was to exclude the possibility that the children chosen originally for the thiamine group were accidentally somewhat superior genetically or otherwise. Reversal of the entire number of sixty pairs was avoided. Since it was known at the orphan-

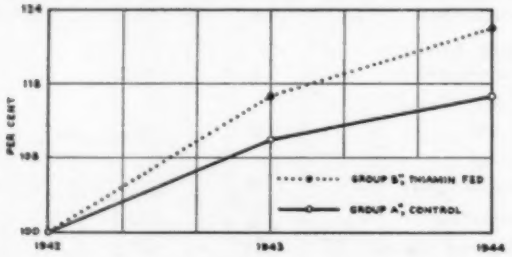

Fig. 4 Average performance of twenty non-reversed pairs in 2 years, eleven activities combined. (Each item is expressed as a percentage of its mean initial score).

age that the children had been arranged in two groups, there was danger that someone would guess a general reversal had occurred and that this would introduce some subjective influence on the results if such a belief became general. Again as in the previous experiments, the full schedule of tests was confined to those pairs of children who could read. There were twenty pairs of children subject to the full schedule of tests which were reversed and twenty pairs which were not reversed, all of whom remained in the institution throughout the 2 years.

The effect of reversal in the second year-long experiment must be considered by comparing the results of the second

year with those of the first. The combined average percentage gains for all eleven activities (not including the memory tests omitted in the measurement of primary grade children) practiced by twenty pairs of children which were not reversed

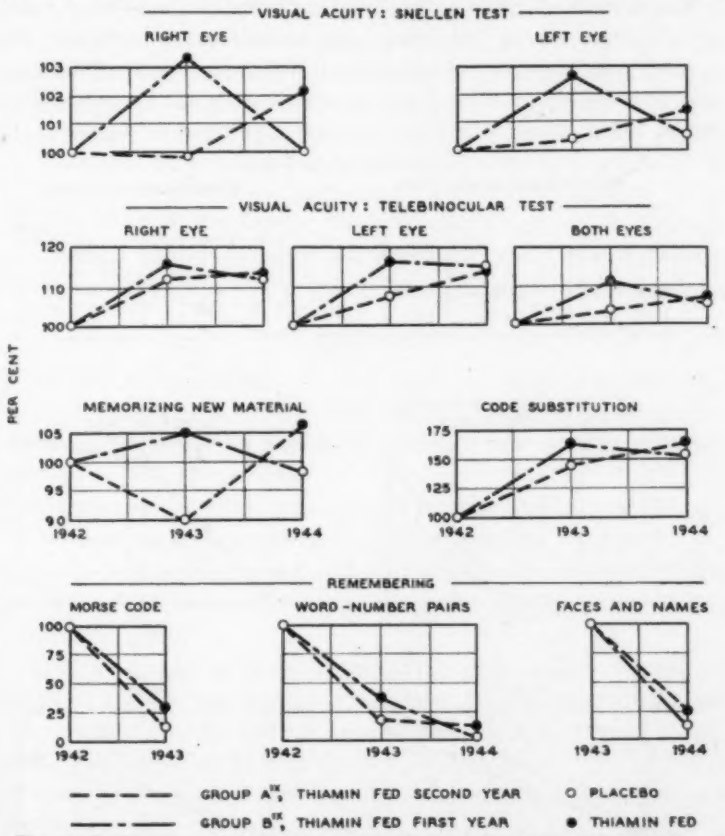

Fig. 5 Performance of twenty reversed pairs showing reversed superiority of gain. (Measures were made at approximately annual intervals).

are shown graphically in figure 4. Trends for the average of each activity separately do not in any case differ greatly from that of the composite shown in figure 4. The vitamin fed children gained an average of 15% in performance the first

year and 22% the second year; the control group gained 10% and 15%, respectively. The reader is referred to the full presentation [1] of the study for further detail which cannot be presented here for lack of space.

Examination of the corresponding tests of the twenty pairs of children whose thiamine supplement was reversed the second year reveals two somewhat different trends. The tests which revealed reversed superiorities of gain are shown in figure 5 and those which do not are presented in figure 6. In

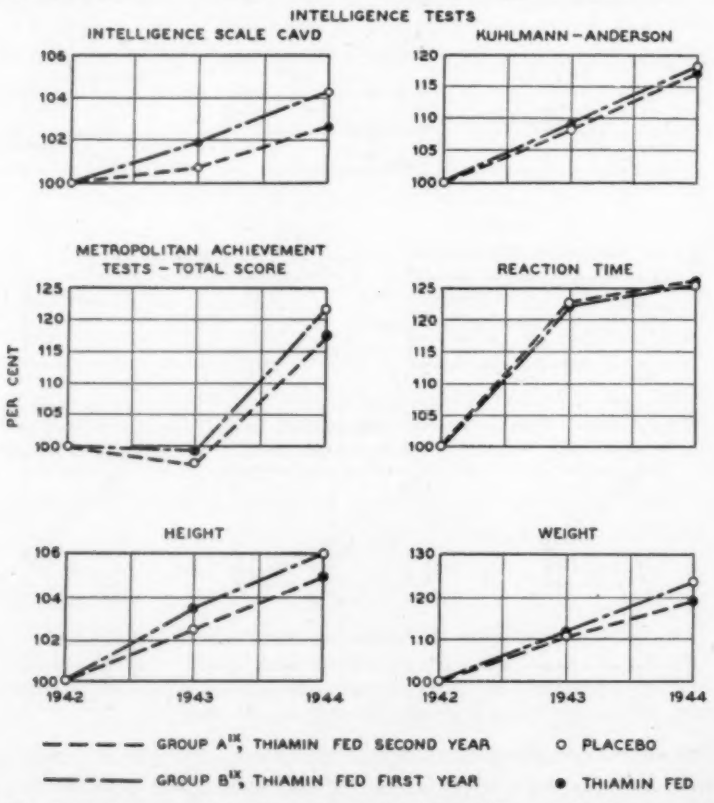

Fig. 6 Performance of twenty reversed pairs in activities not showing reversed superiority of gain. (Measures were made at approximately annual intervals).

the former the gain curves cross following reversal as would be the case if the current intake of thiamine were the controlling factor; in the latter, the curves do not cross. Evidence is wanting of the effect beyond a year's duration of withdrawing thiamine from those who had previously received it. The results suggest that the characteristics referred to in figure 6 change more slowly but more permanently and that the effect of the thiamine taken the previous year held over appreciably into the second year. It is clear that the results of thiamine supplementation are not sufficiently great to be observable over short periods of time with respect to all measures of performance. The cumulative effects throughout a lifetime, however, may nevertheless spell the difference between alert, successful living and a marginal effectiveness.

SUMMARY

During three periods of time between May 1941 and September 1944, the effect was observed of daily supplementation of the dietary of an orphanage with 2 mg of thiamine per child.

Thirty-seven to fifty-five carefully matched pairs of children were used, one member of each pair receiving thiamine and the other an indistinguishable placebo. No participant, child or adult, had knowledge concerning the group to which any child belonged.

Measurements of performance included acuity of vision, skills at games, reaction time, reading, arithmetical processes, memorizing and forgetting, intelligence tests and other recognized measures in current use by psychologists. The schedule of tests was adapted to the length and circumstances of each period.

In the first period of 6 weeks the vitamin fed group made superior average gains in performance in every one of the eighteen test tasks. The individual variations in gains within each group were within the limits of probable error in the case of seven of these tasks.

In the second period of 1 year, fifteen activities were used and the vitamin fed group again surpassed the control group in gains in performance in every activity. The superior gain of the vitamin fed group was statistically significant for eight of the fifteen activities.

In the third period of 1 year, without knowledge of the participants twenty pairs of children were continued with the same regimen as in the previous year while twenty pairs were so reversed that those who had received thiamine now received a placebo and vice versa. The unreversed pairs continued to show superiority of average performance for the thiamine fed group in all eleven tests used; the reversed pairs showed reversals of superior gain in seven activities but failed to show adverse effects of withdrawal of thiamine in intelligence tests, educational achievement, reaction time, height or weight gains.

LITERATURE CITED

HARRELL, R. F. 1943 Effect of added thiamin on learning, Bureau of Publications, Teachers College, Columbia University, New York.

"Student" 1925 New tables for testing the significance of observations. Metron, vol. 5, p. 105.

TAYLOR, C. M. 1942 Food values in shares and weights. Macmillan Company, New York.

STUDIES ON CAROTENOID METABOLISM

VI. THE RELATIVE PROVITAMIN A ACTIVITY OF CAROTENE WHEN INTRODUCED ORALLY AND PARENTERALLY IN THE RAT [1]

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TWO FIGURES

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Although there can be no doubt that β-carotene is the mother substance of vitamin A in the animal body, little is known of the mechanism of such a transformation or of the site in the organism where the change may occur. It is generally considered that an enzyme, carotenase, in the liver is responsible for effecting this change but the experimental evidence substantiating such a hypothesis is exceedingly tenuous. Olcott and McCann ('31) presented the first positive results when it was shown that a slight inflection could be detected in the absorption spectrum at 328 mμ when rat liver slices or liver brei was incubated with a carotene sol for 36 hours. Vitamin A was not isolated, however, and the transformation proceeded only to an extremely limited degree if one is willing to accept such a formation from the spectrophotometric proof offered. In a number of cases there has been a failure to demonstrate any in vitro changes of carotene in the liver. No alterations in this substance could be shown in the liver of the shark (Euler and Euler, '31) or the cat (Rea and Drummond,

[1] The material contained in this paper is part of a thesis submitted by Edwin L. Sexton in partial fulfillment for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Southern California.

[2] Now with The Best Foods, Inc., Buffalo, New York.

'32; Drummond and McWalter, '33). Pariente and Ralli ('31–'32) have reported one successful experiment out of four attempts where the formation of vitamin A from carotene was demonstrated in dog liver. Euler and Klussman ('32) obtained positive results on cow liver as did Wilson et al. ('37) on rabbit liver undergoing anaerobic autolysis.

Some of the discrepancies between the results of different investigators may be related to species variations in the metabolism of the carotenoids, a variability which has long been recognized (Zechmeister, '37). Jensen and With ('39), for example, have found wide differences in the carotene content in the livers of various species. In an extensive study which included 33 mammals comprising 21 species, 41 birds of 36 different species, 4 reptiles from 2 species and also 8 different human specimens, it was found that carotenoids were completely absent in many of the livers examined. Considerable amounts of these polyenes were found in the livers of beasts of prey, birds and the herbivora. Similar discrepancies in the carotenoid content of the blood and tissue fat between different species also have been noticed. Thus, it is known that these pigments are absent from the blood and tissue fat of the rat and pig, but they may be detected in varying amounts in the case of chickens, cattle, horse and men (Zechmeister, '37).

It has previously been reported from this laboratory that a marked decrease in the carotene excretion in milk occurred as a result of the administration of massive doses of vitamin A to cows (Deuel et al., '41); a simultaneous lowering in blood carotene also occurs (Deuel et al., '42). That this behavior is not confined to the cow was shown by a similar reaction in the hen where the yolk carotenoid as well as that in the liver, blood and in the tissue fat was markedly lowered on diets containing large doses of vitamin A (Deuel et al., '43).

The present experiments were designed to determine whether an increased rate of disappearance of carotene could be shown as a result of an augmentation in the amount of carotenase in the livers of rats previously treated with abnor-

mally high doses of vitamin A. It is also possible that an increased amount of lipoxidase, an enzyme first described by Hauge ('35), might be responsible. Since it was found that parenterally injected carotene remained unchanged in the livers of rats for many days, tests on the comparative biological activity of carotene introduced parenterally and orally were made. As a result certain possibilities regarding the site of carotene conversion to vitamin A have been suggested.

METHODS

Rats from our stock colony were kept on a limited intake of vitamin A similar to that employed in preparation for vitamin A bioassays. In the experiments in which the effect of the previous vitamin A intake on the disposal of carotene by the liver was to be followed, weanling rats were divided into five groups and placed on the U.S.P. XII vitamin A-free depletion diet for 28 days. During this interval each group received 0, 30, 450, 900 or 3000 International Units (I.U.) of vitamin A daily which was administered separately from the basal diet. Vitamin A-depleted rats maintained with sufficient vitamin A daily to prevent death but insufficient to clear up the eye symptoms or to produce growth were used for the injection experiments in which the animals were to be sacrificed.

In most cases the carotene employed consisted of 90% β-carotene and 10% α-carotene supplied by General Biochemicals, Inc. In the bioassay experiments pure β-carotene was used.[3] Liver carotene and vitamin A were determined spectrophotometrically on petroleum ether extracts obtained after a 30-minute hot saponification in the dark in which a total of 25 ml of 95% ethanol was used and also 1 ml of 40% aqueous KOH per gram of liver.

In certain experiments where the liver carotene was characterized chromatographically, the livers were frozen in solid CO_2 immediately on being removed from the animals, and powdered in the frozen state by passing through a Wiley mill. Carotene was extracted with methanol without saponi-

[3] This was kindly furnished us by Prof. L. Zechmeister.

fication from this powder, transferred to petroleum ether and chromatographed on a Ca $(OH)_2$ column.[4]

Three carotene preparations were employed. The first sol where carotene was suspended in blood plasma remained perfectly uniform without any loss in carotene when kept for a period of 30 days at 40°C. in the dark. The carotene concentration was adjusted to 60 μg. per milliliter.

A lecithin-stabilized sol was also employed in some of the tests. The carotene and lecithin,[5] dissolved in diethyl ether by shaking, were slowly added to propylene glycol which in turn after evaporation of the ether was mixed with water at 100°C. with vigorous shaking. After standing over night, it was passed through a Buchner funnel. The sol was completely homogeneous and had a concentration of 160 μg per milliliter.

A concentrated carotene solution in cottonseed oil, containing 3000 μg. of carotene per milliliter, was prepared by shaking a diethyl ether solution of carotene with cottonseed oil under nitrogen for 45 minutes, evaporating the ether under reduced pressure and removing undissolved carotene by filtration. The solution was employed in intrasplenic injections. It remained uniform for 3 days or more at room temperature.

The plasma and lecithin sols were well tolerated when introduced orally, intraperitoneally or into the blood stream by cardiac puncture. The oil solution of carotene was satisfactorily administered by intrasplenic injection.[6]

Because of the failure to detect appreciable amounts of carotene in the liver under any conditions where it was fed orally, balance experiments were instituted and the carotene content of the liver was determined after known amounts had been absorbed. In one series of these tests carotene was administered in oil daily over a period of 10 days along with the vitamin A-free diet while in the second series, it was given in natural form as a component of alfalfa flour over a like

[4] We wish to thank Prof. L. Zechmeister for carrying out the chromatographic separation.

[5] Kindly furnished by the American Lecithin Company.

[6] We wish to thank Mr. Frank Cramer who demonstrated this method to us.

period. The latter diet was identical with the vitamin A-free depletion diet except that 25% of the starch was replaced with alfalfa flour. In order to prove that carotene was not destroyed in the gastrointestinal tract, experiments on the excised tract were carried out by a method similar to those on vitamin A reported earlier (Reifman, Hallman and Deuel, '43). In the bioassay tests, rats previously depleted of their vitamin A stores were used.

RESULTS

The effect of previous vitamin A feeding on liver carotene following carotene injection

The carotene and vitamin A concentration in the livers of rats which had previously received various levels of vitamin A daily over a 28-day period following weaning are recorded in table 1 at various periods up to 9 days after the intravenous injection of a carotene sol stabilized in plasma. It should be kept in mind that these and subsequent values for vitamin A

TABLE 1

The carotene and vitamin A content of the livers of rats at various periods following the intravenous injection of a plasma sol containing carotene after the previous administration of vitamin A at different levels over a 28-day period.

PERIOD FOLLOWING INJECTION	PER CENT OF INJECTED CAROTENE RECOVERED [1]					LIVER VITAMIN A IN I.U. PER GM				
GROUP [2]	1	2	3	4	5	1	2	3	4	5
hours										
0.15	13	22	40	29	32	10	10	396	589	1970
2	68 [3]	60	61	58	73	23 [3]	6	368	528	1410
6	69	75	58	56	67	20	14	362	546	1510
12	67	73	58	59 [3]	54	17	16	198	437 [3]	1670
24	64 [3]	66 [3]	54 [3]	60	61 [3]	18	14 [3]	332 [3]	525	1740 [3]
168	39	42	30	38	35	10	11	297	464	1590
216	34	37	35	32	33	8	11	374	405	2060

[1] The average dose of carotene administered was 105 μg.

[2] The previous daily level of vitamin A administration in I.U. was as follows: group 1, 0; group 2, 30; group 3, 450; group 4, 900; group 5, 3000.

[3] Average of two rats. Other results are on single rats.

are not reliable when values of less than about 20 I.U. per gram are involved. The limitations of the method would make it preferable to designate these as "apparent" vitamin A.

It is apparent that no differences obtain over a 9-day period in the rate at which carotene is destroyed in the liver irrespective of the previous level at which vitamin A was administered. It is also evident that no appreciable vitamin A storage took place in the liver when 30 I.U. of vitamin A had been given daily; but with the higher doses the amounts deposited were progressively higher.

The proof of the purity of the carotene isolated from the liver after carotene injection

In order to establish the identity of the carotene isolated from the liver with that injected, the absorption curves were determined on extracts of the original plasma sol injected, and on the samples isolated from the livers 7 and 9 days after injection. That the material is entirely unaltered is indicated by the curves given in figure 1. The points for the two maxima are identical in all three samples. The identity of the sample of carotene isolated from the liver was further established as all-trans-β-carotene by chromatographic separation.[4]

The comparative amounts of liver carotene and vitamin A after parenteral and oral administration of carotene

A further comparison of the effect of injecting carotene by various pathways was carried out on rats previously depleted of vitamin A. Since the livers were already depleted of vitamin A, it was possible to determine whether an accumulation of vitamin A takes place in the liver. These data are summarized in table 2.

Whereas the basal level of liver carotene was practically zero, a considerable amount was found in this organ when carotene was injected by the intravenous or intraperitoneal route as the plasma or lecithin sols or by the intrasplenic route

in the cottonseed oil solution. However, no measureable amount of the liver carotene was present after the oral administration of the carotene sols. The same was true when a diet containing 25% of alfalfa was fed.

There was no increase in the vitamin A content of the liver after the intrasplenic injection of the carotene in oil solution although this value was markedly increased after carotene or

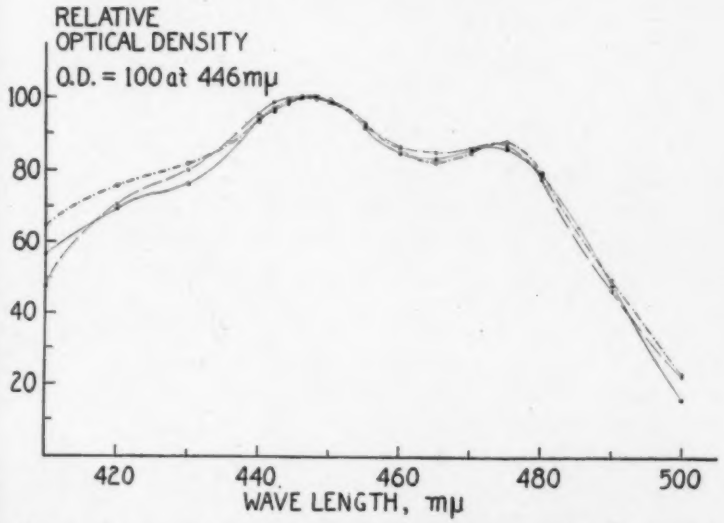

Fig. 1 The absorption curves of carotene extracted from the original sample of plasma-stabilized β-carotene sol (solid line), of carotene isolated from liver after 7 days (dashed line) and 9 days (dashed and dotted lines).

vitamin A was given orally in cottonseed oil or after the alfalfa diet. It seems probable that the low basal levels actually determined for carotene and vitamin A in the liver are within the experimental error of the method or that may represent impurities rather than the actual provitamin and vitamin A. It is probable that these values would be reduced to zero if the liver hydrolysates were purified by chromatographic adsorption.

TABLE 2

Summary table of the carotene and vitamin A content of the livers of rats previously on a vitamin A-free diet after the administration of carotene by several parenteral pathways, of an alfalfa diet, of vitamin A orally and when no supplement was given.

MATERIAL ADMINISTERED	SOLVENT	PATHWAY [1]	NO. OF RATS	QUANTITY ADMINISTERED	ELAPSED PERIOD [2]	LIVER CONTENT	
						Total carotene	Vitamin A
				μg	*hours*	*μg*	*I.U./gm*
Carotene	Plasma	O.	2	104	24	5.7	....
			5	1138	24	6.5	....
		IP.	2	104	17	41.6	....
			4	1138	24	176.8	....
	Lecithin	O.	5	1900	24	9.5	....
		IP.	3	316	24	91.3	....
		IV.	4	316	24	189.9	....
	Cottonseed oil	O.	5	1720	24	6.2	....
			5	2910	24	3.4	13.3
			3	3020 [3]	240	2.9	109.7
		IS.	3	875	24	578	9.1
			5	975	240	529	7.9
Vitamin A	Cottonseed oil	O.	4	1680 [3,4]	240	....	86.6
Alfalfa diet [5]	....	O.	4	2080 [6]	336	7.9	42.4
Vitamin A-depletion diet	....	O.	19	0	...	1.5 (0–5.1)	10.8 (8.4–12.1)

Where no values are reported, no determinations were made.

[1] O., oral; IP., intraperitoneally; IV., intravenously; IS., intrasplenically.

[2] This is period elapsing from first administration of carotene to the time when animal was sacrificed.

[3] Given in two equal divided doses on 2 successive days.

[4] I.U. of vitamin A administered.

[5] Containing 25% alfalfa. Fed over 14-day period.

[6] Total carotene ingested in 14 days. This averages 148 μg daily.

Experiments on absorption of carotene

Since there was no evidence for the accumulation of carotene in the liver after the administration of large amounts of the provitamin by mouth, experiments were undertaken to determine the quantity absorbed from the gastrointestinal tract under a variety of conditions. The summary of these data is recorded in table 3.

TABLE 3

The recovery of carotene from the gastrointestinal tract of rats fed carotene sols, an alfalfa diet, or a control vitamin A-free diet.

CAROTENE			NO. OF RATS	DURATION OF TEST AFTER FIRST CAROTENE ADMINISTRATION	PERIOD OF FASTING		CAROTENE RECOVERED		CAROTENE UNABSORBED
Type of sol	Total fed	Days administered			Before carotene	After carotene	G.i. tract	Feces	
	μg			*days*	*days*	*days*	*μg*	*μg*	%
Oral feeding									
Plasma	104	1	2	1	2	1	20.3	44.2	62.0
	1138	3	5	4	0	0	104	499	53.2
Lecithin	1900	3	5	4	0	0	290	414	37.0
Cottonseed oil	1720	1	2	1	0	1	465	316	45.4
	1720	1	2	1	1	1	467	236	40.9
	1720	1	1	1	2	1	409	272	39.6
	3020	2	3	10	0	0	6.0	1580	52.4
Alfalfa diet	2080	14	4	14	0	0	14.0	924	45.2
Control diet	0	0	5	0	0	0	2.0	9.6	...
Intraperitoneal administration									
Plasma	1138	3	4	4	0	0	48.7	13.8	5.5

Considerable amounts of the carotene fed were still unabsorbed in the g.i. tract 24 hours after the last feeding of the provitamin. Where the feedings of carotene had been continued over several days, a fairly large proportion was lost in the feces. After the administration of 1720 μg. in cottonseed oil,

the loss by this route was of the order of 15%; on the other hand, after flooding the organism with 3020 μg. in cottonseed oil, slightly over 50% was accounted for in the feces over a 10-day period. Since the experiment was continued for 9 days after the last feeding of carotene, it is altogether possible that the carotene may have partially been absorbed and reexcreted. Also, about 45% of the carotene (exclusive of carotenols) fed in the alfalfa diet was excreted in the feces. The control tests on rats fed on a diet, which was carotenoid-free for several weeks, indicates that the quantity of carotene extracted from the gastrointestinal tract and from the feces on a carotene-free diet is inconsequential.

The stability of carotene in the excised gastrointestinal tract

In order to determine if the relatively large quantities of carotene which disappeared in the previous tests recorded in table 3 actually were absorbed and not simply destroyed in the gastrointestinal tract, tests were made to determine whether any destruction could be noted of carotene kept in the excised gut for 24 hours at 37°C. After introducing 2090 μg. of carotene in 1 ml of cottonseed oil directly into the stomachs of anesthetized rats using a stomach tube, the gastrointestinal tracts were removed intact, the esophagi were ligated, the carotene solution was manually forced throughout the alimentary tract and they were placed in the incubator in glass-stoppered flasks for various intervals. The percentages of the original amounts of carotene administered which were recovered after various periods were as follows: 6 hours, 100 and 101%; 12 hours, 90 and 102%; 24 hours, 97, 98, 100, 101%.

These values compare well with experiments with the plasma sol where the extent of the recovery was determined on removing the g.i. tract immediately after the administration of approximately 100 μg of carotene. The values found in several experiments were 100, 102, 105, 106, and 107%.

Bioassay experiments

A series of bioassay tests were made in order to compare the effectiveness of an excess of carotene given orally or intrasplenically and of vitamin A given by the latter route in supplying the vitamin A requirements of vitamin A-depleted rats fed on the U.S.P. XII vitamin A-depletion diet. The supplements were administered in a single dose at the start of the experiments. Five groups of rats were used as follows: group 1, negative controls which received no supplement; group 2, carotene given orally; group 3, carotene given intrasplenically; group 4, vitamin A given intrasplenically; group 5, a continuation of the survivors of group 3, with carotene given orally on the forty-sixth day. Some rats in each group except the negative controls were sacrificed during the course of the experiment to ascertain the level of carotene and vitamin A in the liver. All animals in the negative control group had died before any rats in the other groups were sacrificed. The remaining animals were continued on the vitamin A-free diet until death occurred. The rats were weighed at approximately 5-day intervals. A summary of the data on growth and period of survival is given in table 4.

Although the dosages of the carotene and vitamin A are not identical, they were far in excess of what could be stored and were calculated to give the maximum effect for a single dose. That this supposition is justified is indicated by the identity of the results of groups 2 and 4 where the same maximum increase in weight resulted, the day at which the maximum was reached is practically the same and the period of survival is of the same order. The dose of carotene given orally was higher than that given parenterally to compensate for the carotene lost in the stools in the former case. With the group which received carotene intrasplenically, the maximum weight increase was smaller and occurred on the thirty-fifth day. By the forty-sixth day on which a supplementary dose of carotene was given orally, three of the group had already died and the rest had all lost considerable weight from their

TABLE 4

Summary table showing the maximum gain in weight and average length of life of vitamin A-depleted rats on a vitamin A-free diet after a single dose of carotene or vitamin A.

GROUP NO.	SUPPLEMENT ADMINISTERED	NUMBER OF RATS		AVERAGE DOSE GIVEN	DEPLETION WEIGHT	MAXIMUM INCREASE IN WEIGHT [2]	AVERAGE DAY OF DEATH [3]
		Start	Killed or died before 46 days [1]				
					gm	*gm*	
	Male rats						
1	Negative control	9	9	0	101.4	....	19.4(9)
2	Carotene orally	7	2(1)	1480 μg	100.4	104.6(50)	66.8(6)
3	Carotene intra-splenically	4	1(2)	592 μg	94.0	33.7(35)	[4]
4	Vitamin A intra-splenically	6	3	415 I.U.	96.8	108.0(50)	84.0(3)
5	Carotene orally	1	..	1500 μg	125 [5]	54 (30)	46 (1)
	Female rats						
1	Negative control	6	6	0	103.5	....	15.3(6)
2	Carotene orally	6	3	1480 μg	98.7	72.0(55)	90.3(3)
3	Carotene intra-splenically	9	3(1)	592 μg	101.3	36.5(35)	[4]
4	Vitamin A intra-splenically	8	4	415 I.U.	105.2	74.5(60)	104.5(4)
5	Carotene orally	5	..	1500 μg	148.0 [5]	36.4(44)	82.6(5)

[1] The values in parentheses are the additional number of rats which died before the forty-sixth day.

[2] The figure in parentheses indicates the day on which the maximum increase in weight was reached.

[3] Excluding those killed for the analysis of the livers. The number of rats in the average is included in parentheses.

[4] Continued as group 5.

[5] Because the rats of group 3 which died or had been killed before starting as group 5 had lost more than the survivors, there is an apparent discrepancy between the average maximum weight increase in group 3 and the depletion starting weight of group 5. However, all the survivors in group 3 had started to lose weight before starting in group 5.

maximum levels. Eye symptoms of avitaminosis A had reappeared.

A comparison of the growth curves is shown in figure 2.

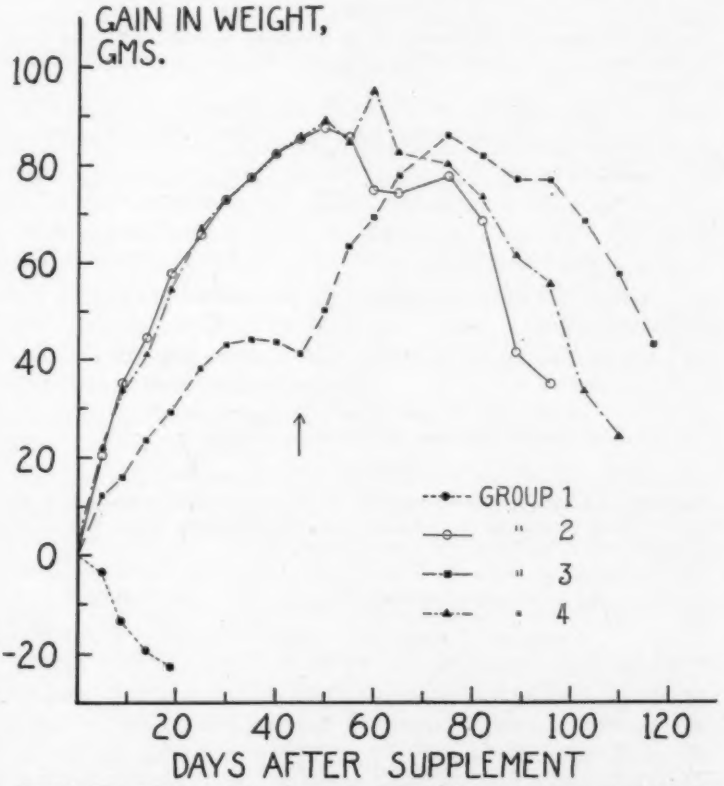

Fig. 2 The gain in weight of vitamin A-depleted rats which received no vitamin A supplement (group 1), or a single supplement at 0 days of carotene orally (group 2), of carotene intrasplenically (group 3), followed by carotene orally at 46 days as indicated by arrow (group 3 only), or vitamin A intrasplenically (group 4).

Table 5 gives the values for liver carotene for the rats which were killed on the forty-sixth day or died earlier in groups 1, 2 and 4; table 6 also gives additional data on group 3.

The carotene isolated from the livers of rats 46 days after the intrasplenic injection was proved to be all-trans-β-carotene by its absorption curve and by chromatographic separation.[4]

TABLE 5

The average carotene and vitamin A in the livers of male and female rats which died before or were killed on the forty-sixth day after the supplements were administered.

GROUP NO.[1]	AVERAGE DOSE GIVEN	LIVER CAROTENE IN μG		LIVER VITAMIN A IN I.U./GM	
		Individual determinations	Average	Individual determinations	Average
1	0	0.4, 0.4, 0.7, 1.1, 1.1, 1.1, 1.4, 1.9, 2.7, 2.7	1.3	6.7, 7.5, 8.0, 8.1, 8.2, 9.5, 9.9, 10.3, 11.4, 12.3, 20.6	10.2
2	1480 μg	0.8, 1.9, 2.3, 4.2, 4.6, 6.5	3.4	5.9, 6.5, 10.9, 11.9, 17.8, 30.2	13.9
4	415 I.U.	3.1, 4.2, 4.6, 5.1, 5.1, 5.5	4.9	10.8, 13.5, 15.9, 16.7, 24.3, 25.8, 26.5	19.1

[1] The group numbers are the same as in table 4 and figure 2.

TABLE 6

Summary of body weight changes and of the carotene and vitamin A in the livers of rats previously injected with carotene intrasplenically. These died on the forty-fourth day or were killed on the forty-sixth day.

RAT NO.	SEX	BODY WEIGHT IN GM			LIVER	
		Depletion	Maximum [1]	Final [2]	Carotene	Vitamin A
					μg	I.U.
30	M	108	127(25)	112(45)	141	18.4
31	F	92	125(35)	115(45)	171	17.4
33	F	93	135(25)	121(45)	76	12.4
41	F	106	144(25)	126(45)	77	8.3
39	M	90	133(35)	128(40)	185 [3]	41.5 [3]
42	F	119	128(14)	91(40)	214 [3]	98.8 [3]
Average [4]					116	14.1

[1] Figures in parentheses are the first day the maximum weight was attained.

[2] Figures in parentheses are the day of the last weight before the rat died or was killed.

[3] Animals died on forty-fourth day. Because post mortem autolysis of liver had started before the organ was removed, these results are not included in average.

[4] Average of first four rats only.

No appreciable amount of vitamin A over the basal level was found after intrasplenic injection of carotene except in the case of rats 39 and 42 which died of vitamin A deficiency. Had this amount of vitamin A been available earlier, it presumably should have been sufficient to alleviate the deficiency symptoms.

DISCUSSION

Carotene was not found in the liver of the rat even after large amounts were administered as the plasma or lecithin sol or as a cottonseed oil solution provided the oral route was used. Moreover, carotene could not be detected in the liver of rats which had received a diet containing 25% of alfalfa flour for 14 days. That the carotene administered orally must have been absorbed in large part was indicated by absorption experiments as well as by the proof that it was not destroyed in the excised gut over a 24-hour period. Further proof that the carotene had been metabolized is afforded by the fact that increased levels of vitamin A were found in the liver over those of the control animals.

On the other hand, carotene is deposited in the liver to a large extent when given parenterally either as a plasma or a lecithin sol or in cottonseed oil solution. Moreover, it is retained in the liver over long periods of time since relatively large amounts of stereochemically pure carotene were found 46 days after the injection.

In spite of the fact that relatively large amounts of carotene may remain in the liver after its parenteral injection, such carotene cannot be used as a source of vitamin A. The livers of two rats which died on the forty-fourth day after intrasplenic injection of carotene had 185 and 214 μg of carotene, respectively. This quantity would be sufficient to cause limited growth over more than a year if administered at a level of 0.5 μg daily as we have obtained a minimum growth for 28 days in bioassay tests at that level (Deuel et al., '45).

When carotene is introduced intrasplenically, it may be argued that it is immediately taken up by the Kupfer cells on reaching the liver where it is retained as any foreign substance

would be (Maximow and Bloom, '42). However, vitamin A when injected by the same pathway is utilized. Under normal conditions when carotene is given orally one should expect it to reach the liver from the gastrointestinal tract in part or at least also by the portal route. Why should it not under these conditions also remain immobilized in the liver?

In the first place, it is possible that it may be metabolized if it reaches the liver in low concentrations while if administered in large amounts, it is deposited in the Kupfer cells and can only with difficulty be utilized if at all. However, the fact that there is no indication of a better utilization when administered in low doses as compared with high concentrations would seem to speak against this idea. Secondly, the possibility exists that carotene may normally be largely absorbed by the lymphatics and then reach the liver first through the hepatic circulation, thus avoiding the portal route. However, in the experiments where the plasma sol was injected into the heart, this pathway should be duplicated and a large proportion of the carotene still remained inactivated in the liver. Finally, carotene might under normal conditions be carried to the liver in small amounts as a protein conjugate. Such a combination apparently exists in the case of some species of animals[7] although it has not been detected in rat blood (Zechmeister, '34).

It is possible that if such a conjugated material is formed, it may be removed by the liver of the rat and converted to vitamin A at a rate greater than that at which it enters the blood stream. None of these possibilities offer an entirely satisfactory explanation of the differences obtained for the behavior of parenterally- and orally-introduced carotene.

The experiments confirm those reported earlier by Lease et al. ('42), who found that the amount needed for growth was ten to one hundred times as great when aqueous carotene sols of oil solutions of carotene were given intraperitoneally

[7] One of us (J.W.M.) has shown in confirmation of other workers that the carotenoids in cow's blood migrate in the Tiselius apparatus with the beta globulin fraction.

or subcutaneously as contrasted with the quantity required when the products were administered orally. Deficiency symptoms and death occurred when considerable amounts of carotene were still in evidence in the liver, in lymph nodes in the peritoneal cavity and at the sites of injection. On the other hand, vitamin A was well utilized when given parenterally.

The present experiments not only confirm Lease et al. ('42) but they extend the observations in demonstrating that the deposited carotene is unaltered and that it is stereochemically identical with the injected product. Our experiments also demonstrate that no fundamental disorder in carotene metabolism develops in rats having these abnormal deposits of carotene. Otherwise, carotene administered orally would not correct the deficiency symptoms and cause a resumption in growth.

A further difference in the behavior of carotene administered parenterally as contrasted with that given orally is that it fails to give rise to any vitamin A in the liver. The average values of 7.9 and 9.1 I.U. obtained for apparent vitamin A per gram of liver are within the range found in the livers of vitamin A-depleted control rats (8.4–12.1). After the oral administration of carotene in oil, a mean of 109.7 I.U. of vitamin A was present while after the alfalfa diet 42.4 I.U. were found per gram of liver. Lease et al. ('42) also were unable to demonstrate vitamin A storage in the liver after prolonged parenteral injection.

It is hard to reconcile the data reported here with the current conceptions of carotene metabolism. If carotene is normally transformed to vitamin A in the liver, then one should be able to demonstrate such an enzymatic system in a more convincing manner than has hitherto been possible. One should also be able to find appreciable amounts of carotene in the blood and definite amounts in the liver unless it is assumed that the enzymatic change is so rapid that it would prevent any accumulation of the provitamin. Finally, the almost complete ineffectiveness of parenterally introduced carotene to effect any prolonged remission of avitaminosis A symptoms even when considerable amounts of pure carotene

are still present in the liver would also argue against the liver as the site of activation of the carotene. Coupled with this observation is the fact that no vitamin A is deposited in the liver after such parenterally introduced carotene while considerable amounts are stored after the oral administration of comparable amounts of the provitamin.

One explanation for these phenomena would be that carotene is transformed into vitamin A in the rat before reaching the blood stream. A possible site for the transformation of carotene to vitamin A might be in the intestinal wall. The change does not take place within the lumen as no destruction was found in carotene present in the excised gut over a 24-hour period at 37°C. Considerable amounts of carotene also accumulate in the wall of the intestine during absorption (Shaw and Deuel, '44) so the transformation there cannot be an immediate one.

In earlier experiments on carotene absorption, a complete absorption of this provitamin was indicated when given in oil solution (Shaw and Deuel, '44). After administration of an average of 3640 μg of carotene in oil, 3110 μg had disappeared from the lumen of the gut by 18 hours. Over 50% (2040 μg) was shown to be present in the wall of the intestine at this time. This was in contrast with the results of Kemmerer and Fraps ('38) and a more recent report of Fraps and Meinke ('45). A possible explanation of these discrepancies is that our earlier tests were carried out with rats previously fasted 2 days or more with the result that no feces were produced or if so only at the start of the test. In the experiments of Fraps, the animals were fed during the absorption tests which resulted in carotene being mechanically carried out. When fed as a component of vegetables, it is also probable that an incomplete digestibility may be ascribed to an incomplete breakdown of the vegetable fibers. In the present tests, considerable portions of carotene were excreted in the feces but this is partly to be ascribed to the fact that the rats were fed instead of fasted previous to and during the carotene absorption. In the series where carotene was given in oil to fasted

rats, less than 15% was excreted in the feces. This incomplete absorption in the present tests may also be because smaller rats were employed than in the former experiments.

SUMMARY

1. No relation was found between the rate at which carotene was destroyed after parenteral injection in the rat and the previous level of vitamin A administration. Vitamin A had been given at levels of 0, 30, 450, 900 or 3000 I.U. daily for 28 days previous to the carotene administration.

2. No carotene could be demonstrated in the livers of rats after oral administration of carotene as a plasma or lecithin sol, or in a cottonseed oil solution or after feeding a diet containing 25% of alfalfa for 14 days. Increased levels of vitamin A were observed in the livers under such conditions.

3. Carotene was deposited in the liver after parenteral injection. It was still present in considerable amounts 46 days after the injection and it was shown by spectrophotometric measurements and by chromatographic separation to be all-trans-β-carotene.

4. No increase in vitamin A could be demonstrated after the parenteral injection of carotene.

5. When an excess of carotene was administered orally or an excess of vitamin A was injected intrasplenically in a single dose to vitamin A-depleted rats which were then continued on a vitamin A-free diet, identical growth responses were obtained, and the times when the maximum weight was attained were similar. The average length of survival without additional vitamin A was also approximately the same. On the other hand, after a similar excess of carotene was injected intrasplenically, only a slight growth was obtained and the animals died with the livers still containing large amounts of β-carotene. The quantities of carotene still remaining in the liver at the time of death were sufficient to have maintained the animals well over a year if given in divided doses orally.

6. No intrinsic impairment in carotene metabolism was found in rats showing signs of avitaminosis-A after intra-

splenic injection of carotene since they were able to utilize this provitamin when given orally.

7. No carotene was destroyed in the excised gastrointestinal tract of rats kept at 37°C. for 24 hours.

8. The possibility is suggested that the conversion of carotene to vitamin A may be an extra-hepatic function in the rat. The wall of the intestine is suggested as a possible site of such transformation.

LITERATURE CITED

DEUEL, H. J., JR., N. HALLIDAY, L. F. HALLMAN, C. JOHNSTON AND A. J. MILLER 1941 The production of high vitamin A milk by diet. J. Nutrition, vol. 22, p. 303.

DEUEL, H. J., JR., L. F. HALLMAN, C. JOHNSTON AND F. MATTSON 1942 The effect of a high vitamin A intake on the blood and milk carotene of Holstein and Guernsey cows. J. Nutrition, vol. 23, p. 567.

DEUEL, H. J., JR., M. C. HRUBETZ., F. H. MATTSON, M. G. MOREHOUSE AND A. C. RICHARDSON 1943 Studies on carotenoid metabolism. IV. The effect of vitamin A intake on the carotenoid and vitamin A content of the eggs, liver, blood, and body fat of hens. J. Nutrition, vol. 26, p. 673.

DEUEL, H. J. JR., E. R. MESERVE, C. H. JOHNSTON, A. POLGAR AND L. ZECHMEISTER 1945 Re-investigation of the relative provitamin A potencies of cryptoxanthin and β-carotene. Arch. Biochem., vol. 7, p. 447.

DRUMMOND, J., AND R. MCWALTER 1933 About the biological relation between carotene and vitamin A. Biochem. J., vol. 27, p. 1342.

EULER, H. V., AND B. V. EULER 1931 Zur Kenntnis der Leberöle von Fischen and Vögeln. Svensk. Kem. Tid., vol. 43, p. 174.

EULER, H. V., AND E. KLUSSMANN 1932 Carotene and vitamin A. Svensk. Kem. Tid., vol. 44, p. 223.

FRAPS, G. S., AND W. W. MEINKE 1945 Digestibility by rats of α-, β-, and Neo-β-carotenes in vegetables. Arch. Biochem., vol. 6, p. 33.

HAUGE, S. M. 1935 Evidence of enzymatic destruction of the vitamin A value of alfalfa during the curing process. J. Biol. Chem., vol. 108, p. 331.

JENSEN. H. B., AND T. K. WITH 1939 Vitamin A and carotenoids in the liver of mammals, birds, reptiles and men with particular regard to the intensity of the ultraviolet absorption and the Carr-Price reaction of vitamin A. Biochem. J., vol. 33, p. 1771.

KEMMERER, A. R., AND G. S. FRAPS 1938 The digestibility of carotene by rats and chickens. J. Nutrition, vol. 16, p. 309.

LEASE, J. G., E. J. LEASE, H. STEENBOCK AND C. A. BAUMAN 1942 The comparative physiological value of injected carotene and vitamin A. J. Lab. and Clin. Med., vol. 27, p. 502.

MAXIMOW, A. A., AND W. BLOOM 1942 Textbook of Histology, 4th Ed. W. B. Saunders Co., Philadelphia, p. 428.

OLCOTT, H. S., AND D. C. MCCANN 1931 Carotenase. The transformation of carotene to vitamin A in vitro. J. Biol. Chem., vol. 94, p. 185.

PARIENTE, A. C., AND E. F. RALLI 1931–32 Presence of carotenase in the liver of the dog. Proc. Soc. Exp. Biol. Med., vol. 29, p. 1209.

REA, J., AND J. DRUMMOND 1932 Zur die Bildung von Vitamin A aus Carotin im tierischen Organismus. Z. Vitamininsforsch., vol. 41, p. 177.

REIFMAN, A. G., L. F. HALLMAN AND H. J. DEUEL, JR. 1943 The effect of concentration on the absorption of vitamin A. J. Nutrition. vol. 26, p. 33.

SHAW, R. W., AND H. J. DEUEL, JR. 1943 Studies on the absorption of carotene. J. Nutrition, vol. 27, p. 395.

WILSON, H. E. C., B. AHMAD AND B. N. MAZUMDAR 1937 Transformation of carotene into vitamin A in liver autolysates. Indian J. Med. Res., vol. 25, p. 85.

ZECHMEISTER, L. 1934 Carotinoide. Julius Springer, Berlin.

———— 1937 Die Carotinoide im tierischen Stoffwechsel. Ergeb. Physiol., biol. Chem. exp. Pharmakol., vol. 39, p. 117.

GROWTH AND FOOD PREFERENCE OF RATS FED A LACTOSE-DRIED MILK RATION CONTAINING BUTTER FAT OR CORN OIL[1, 2]

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ONE FIGURE

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In a series of investigations on the nutritive value of butter and vegetable fats, Boutwell et al. ('43 a, b; '45) and Geyer et al. ('43) came to the conclusion that when lactose was the sole carbohydrate of the diet butter fat promoted better growth of weanling rats than did vegetable fats. The effect was more pronounced on diets containing high levels of lactose. When other carbohydrates or carbohydrate mixtures were used, this difference was not observed. The inferiority of corn oil in the lactose-containing rations could be reduced by the addition of vitamins of the B-complex. Zialcita and Mitchell ('44), and Deuel et al. ('44, '45) did not find butter fat to cause greater gains in weight than did vegetable fats, but their rations did not contain added lactose.

The possibility that there are differences in the nutritive value of these different fats has created so much interest and the conclusions appear to be in such an unsettled state (Food and Nutrition Board, '43), it was thought desirable to gain further information on the problem. In the present experiments it was decided to employ butter fat and corn oil rations used by investigators working at the University of Wisconsin. A study of food preference also was made.

[1] Contribution no. 303, Department of Chemistry.

[2] Supported in part by a grant from the American Dairy Association.

METHODS USED

Growth studies

The strain of rats and rations selected for these experiments were the same as used in some investigations by Geyer et al. ('43).[3] Except for experiments V-A and V-B, the rats were obtained from Sprague-Dawley, Madison, Wisconsin; 40–45 gm males weaned at 20 days of age were specified. The rats used for experiments V-A and V-B were from the colony at this laboratory, the original stock having come from the above source.

The rations consisted of the following: ether-extracted skim milk powder, 50 parts; lactose (milk sugar, U.S.P. Merck), 20 parts; and either butter fat or corn oil, 30 parts. Minerals were added so that 10 gm of the complete ration contained 1.5 mg of elemental iron, and 0.15 mg each of elemental copper and manganese. To each 100 gm of fat were added, carotene,[4] 2 mg; α-tocopherol, 8 mg; calciferol,[5] 0.05 mg; and 2-methyl, 1, 4-naphthoquinone, 0.75 mg. Spray-dried skim milk powder was obtained from a plant in Northeastern Kansas. Unsalted, sweet-cream butter was made from cream obtained from the College Dairy; the fat was separated by melting the butter and careful decantation from the water and curd. Corn oil[6] was obtained from a local market.

In order that possible variations in ingredients might be checked, rations of the same composition, prepared at the University of Wisconsin, were used for experiment IV-C.[7]

Weanling rats obtained from the dealer were distributed by weight into the experimental groups; those raised in this laboratory were distributed by litter, sex, and weight. The rats were placed in individual cages with wire floors. Droppings and any wasted food fell onto papers placed in pans beneath the cages. The animal room was maintained at 26–27°C. Each rat was provided a little more food than would be

[3] R. K. Boutwell 1944 Personal communication.

[4] β-carotene, 90%; α-carotene, 10%.

[5] Kindly supplied by E. I. du Pont de Nemours and Company.

[6] Mazola.

[7] We are indebted to R. K. Boutwell for supplying these rations.

consumed before the feeding on the following day. Rations were mixed at not longer than 2-week intervals, kept in tightly-closed jars in the refrigerator, and each day only the feed needed to fill the food cups was removed.

The rats were weighed each week. Records were kept of the weight of ration fed and of any wasted food dropping onto papers beneath the cages. Water was provided ad libitum.

Food preference

The rats used for a study of food preference previously had been used on both growth and maze-learning experiments. (A report on the latter is in preparation.) During the maze-learning trials a restricted feeding schedule was followed which allowed continual growth but resulted in the rats being motivated by hunger during the tests. At the completion of these learning trials the rats had been on one of the experimental diets from 7 to 10 weeks. Food preference was tested by placing the rats in the entrance to a short runway, at the opposite end of which were small food cups, one containing the corn oil ration, the other containing the butter fat ration. In experiment II-B the food cups were alike, and their positions were interchanged in a random manner. For the other two experiments the corn oil ration was placed in a black cup and the butter fat ration in a white cup of the same design. The cups were kept in the same positions throughout the experiment to enable the rat to learn the location of the food of its choice, if it had such. Except for the small amount of food obtained from the cups during the experiment, the rats were maintained on the restricted feeding of either the butter fat ration or the corn oil ration used during the maze-learning trials.

Twice the rats were placed in the entrance to the runway and allowed to explore the food cups and become oriented to the new situation. On the following days each rat was given two trials in which it was allowed to select and eat the chosen food; records were kept of the first three choices made. A choice was recorded only when the rat ate some of the food

from the cup; nothing was recorded if the rat merely explored and turned away from the food. When not as many as three choices were made, the food selected and eaten for a period of 15 seconds was recorded as the last choice. After the rats had eaten for 15–20 seconds, they were removed from the runway. It is believed this technique enabled the rat to select freely whichever food appeared the more attractive. The duration of the tests was: experiment II-B, 4 days; experiment III, 6 days; and experiment IV-B, 9 days. In each experiment twelve rats were used, half previously having been on each ration.

RESULTS

Growth studies

The growth studies are summarized in table 1. In all cases the rats fed the butter fat ration made greater average gains in weight and also ate more of the food. In four experiments, II-B, IV-A, V-A, and V-B, the gains were significant at either the 1% or 5% level of confidence. (t-test, Lindquist, '40). Likewise, the difference in average food consumption was significant for experiments IV-A, V-A, and V-B. An analysis of the average efficiency of conversion of food to body tissue showed that male rats fed for 6 weeks in four different experiments utilize either food for growth to a similar degree of efficiency, all values being between 39.6 and 43.7. Females did not appear to utilize the ration as efficiently as the males, although similar gains were made on both rations. Rats fed for 2½ or 3 weeks utilize the ration more efficiently than when fed for a total of 6 weeks.

Although significant average differences in food consumption were found in only three experiments (probably due to individual differences in rats), the gain in weight made by each individual is related to the intake of either ration. In figure 1 this relationship of food consumed to gain in weight has been shown for all the male rats fed for a total of 6 weeks. A plot of the data for females has not been included; it was similar, except for a small shift in the values due to the smaller gains in weight per gram of food consumed.

Smaller average gains, significant at the 1% level, were made on food obtained from the University of Wisconsin laboratory, but the conversion to body tissue did not differ appreciably from that found in a simultaneous experiment

TABLE 1

Distribution of the rats, food consumed, gains in weight and average efficiency during growth studies.

EXPERIMENT NO.	AGE AT START OF EXPERIMENT AND SEX	DURATION OF EXPERIMENT	RATION	RATS ON EACH RATION	AV. WT. AT START	AV. GAIN IN WEIGHT	AV. WT. OF FOOD CONSUMED	AVERAGE EFFICIENCY (GM GAIN/GM CONSUMED) × 100
	days	*weeks*		no.	*gm*	*gm*	*gm*	
II-B	21 (M)	2½	Corn	6	40	57	110	51.8
			Butter	6	40	66	113	58.5
IV-A	22 (M)	3	Corn	12	42	59	121	48.8
			Butter	12	42	71	144	49.4
II-A	21 (M)	6	Corn	6	40	159	367	43.3
			Butter	6	40	164	376	43.6
IV-B	22 (M)	6	Corn	6	41	153	350	43.7
			Butter	6	42	168	384	43.7
IV-C	22 (M)	6	Corn [1]	6	41	131	307	42.6
			Butter [1]	6	41	135	342	39.6
V-A	21 (M)	6	Corn	15 [2]	40	121	300	40.4
			Butter	15	40	143	332	43.1
V-B	21 (F)	6	Corn	11 [2]	41	105	280	37.5
			Butter	11	42	116	317	36.6

[1] Feed prepared at Wisconsin University.

[2] Litter mates raised at K.S.C.; all other rats from Sprague-Dawley.

(IV-B) using rations prepared in this laboratory. The difference in weight gains indicates a difference in the ration even though prepared according to the same directions. Since the effect was observed on both the corn oil and the butter fat rations, the powdered milk may have been the variable ingredient. However, the fact that the rations prepared in this

laboratory were fresher may have influenced the results obtained. Probably this was not an important factor, since the Wisconsin rations were kept in a refrigerator and rancidity was never detected.

Diarrhea affected many rats, except in experiments V-A and V-B. The condition almost always disappeared in from 5–15 days, generally clearing up a little more rapidly in the

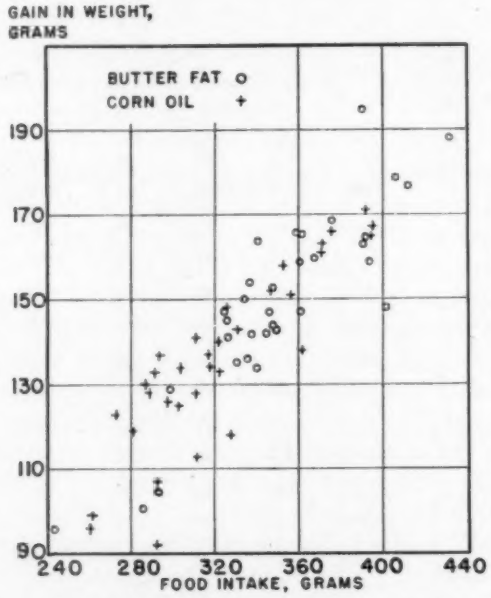

Fig. 1 Scatter diagram showing the relation of gain in weight to food intake for male rats fed butter fat or corn oil rations for 6 weeks.

rats fed the butter fat ration. Rats with diarrhea were not discarded as was done by Deuel et al. ('44), since there is evidence that this condition may possibly be associated with lactose diets (Ershoff and Deuel, '44; Whittier et al., '35). In the entire series of experiments only one animal had to be discarded because of death. This rat appeared entirely normal the previous day. The apparent difference found in the effici-

ency of conversion of the rations to body tissue by rats fed for only 2½ weeks (experiment II-B) may have been due to diarrhea which affected many of the animals. The condition cleared up more rapidly for the rats fed the butter fat ration but remained till near the end of the experiment in rats fed the corn oil ration.

The fur coats of rats on the experimental rations were slightly rougher than those of colony rats fed a complete stock ration, but no differences were apparent among animals on the two experimental rations. A few rats developed a mild alopecia on the posterior part of the abdomen; the condition was not general enough to associate with either diet or experimental group.

Food preference

The first and final (or third) choices of rations in the food-preference experiments are recorded in table 2. In experiments II-B and III the rats showed a tendency to prefer the food previously received; particularly is this true for the rats

TABLE 2

Food choice of rats fed butter fat and corn oil rations.

EXPT. NO.	PREVIOUS RATION	FIRST CHOICE		LAST CHOICE [1]	
		Corn oil	Butter fat	Corn oil	Butter fat
II-B	Corn oil	26	22	28	20
	Butter fat	18	30	14	34
III	Corn oil	34	38	45	27
	Butter fat	15	57	18	54
IV-B	Corn oil	57	51	55	53
	Butter fat	75	33	83	25
Total	Corn oil	117	111	128	100
	Butter fat	108	120	115	113

[1] The food the rat selected and ate for 15 seconds; or if the rat did not eat a selected food for 15 seconds, the third choice was recorded. In some cases the first and last choices are the same, and it is recorded under both headings.

fed the butter fat ration. The results on experiment IV-B are difficult to understand, since the rats previously fed the butter fat ration exhibited a decided preference for corn oil. It is unlikely that anything was seriously wrong with the ration, for the animals previously fed corn oil selected the butter fat ration almost as frequently as they did the corn oil. Furthermore, a new batch of feed was tried with the same results.

While some rats had (or developed) a preference for one or the other ration, learning its location, going immediately to it, and eating it for at least 15 seconds, other rats appeared to be indifferent, sampling first one and then the other ration. A further indication that the rats preferred the food previously received is obtained from the animals showing an exclusive food preference on the last 2 days of the respective experiments. It was found that five "butter" rats preferred the butter fat ration and two the corn oil ration, while seven "corn oil" rats preferred the corn oil ration and three the butter fat ration.

DISCUSSION

The fact that in each experiment greater average gains in weight were made on the butter fat rations (significant in four cases) would indicate there is a difference in response when the two fats are incorporated in lactose-skim milk diets. But factors as yet unidentified lead to results which in repeated experimentation are not always reproducible. The differences in intestinal flora and the requirements for the B-vitamins have been suggested as possible causes of differences in response of rats on corn oil and butter fat in rations containing lactose (Boutwell et al., '43; '45). Perhaps variations in the development of the flora, even when rats of the same strain are fed rations made from the same ingredients, are the cause of a variable response from one experiment to another. Comparison of the growth response made on the rations prepared at the University of Wisconsin and the rations prepared in this laboratory indicates another possible reason why results of studies of the nutritive value of fats do not always agree.

A further explanation for the different conclusions which appear in the literature is that no two groups of investigators have published results based on the same experimental rations. It should be noted that these results agree more closely than do any previous studies with those reported by the Wisconsin investigators who used the same strain of rats and high lactose diets.

Although other investigators also have shown that rats consume more of a butter fat ration than of a corn oil ration, opinions differ concerning the reason. Deuel and Movitt ('44) suggest it is due to the fact that rats prefer a butter flavor, while Boutwell et al. ('44) believe the effect is due to a superiority of the ration not associated with flavor.

The technique employed in the present study of food preference differs from other studies in that the rats were motivated by hunger during the tests, but a priori the present technique is not less valid for determining food preference. Although on the experimental diets for 7 to 10 weeks, the hungry rats did not recognize the butter fat ration as superior to the corn oil ration when offered a choice. Likewise, the food containing the natural butter flavor was not selected more frequently by the rats previously fed on corn oil, as would be expected if flavor determined the choice of ration. But a comparison of the results on this study to others wherein ad libitum feeding was practiced brings up a question whether the two rations might appear equal for satisfying immediate needs, although appetite remains longer for butter fat, or this ration produces a physiological condition in the animal which stimulates greater consumption. Thus in ad libitum feeding, this might account for the greater average gains in weight made on certain rations containing butter fat.

SUMMARY

1. All groups of rats fed ad libitum on butter fat rations made greater average gains in weight than those fed corn oil rations. The results were significant in four of the seven experiments.

2. All groups of rats fed butter fat showed a greater average consumption of the ration than did those fed corn oil. The results were significant in three of the seven experiments. The gain in weight made on either ration was related to the quantity of food the rat consumed.

3. The average efficiency of conversion of the food to body tissue over a 6-week period was similar for both the butter fat ration and the corn oil ration.

4. When given a choice, hungry rats previously fed corn oil exhibit no preference for the butter fat ration over the corn oil ration. The results in these experiments were not found to be consistent for the rats fed the butter fat ration.

5. Rations of the same composition, made of ingredients from different sources, did not cause the same growth response when used in simultaneous feeding experiments.

LITERATURE CITED

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1943 a Further studies on the growth-promoting value of butter fat. J. Dairy Sci., vol. 26, p. 429.

———— 1943 b Further studies on the comparative value of butter fat, vegetable oils and oleomargarines. J. Nutrition, vol. 26, p. 601.

———— 1944 Effect of flavor on nutritive value of fats. Proc. Soc. Exp. Biol. Med., vol. 55, p. 153.

———— 1945 Studies on the interrelation of fats, carbohydrates, and B-vitamins in rat nutrition. Arch. Biochem., vol. 7, p. 143.

DEUEL, H. J., JR., AND E. MOVITT 1944 Studies of the comparative nutritive value of fats. III. The effect of flavor on food preference. J. Nutrition, vol. 27, p. 339.

———— 1945 Studies on the comparative nutritive value of fats. V. The growth rate and efficiency of conversion of various diets to tissue in rats weaned at 14 days. J. Nutrition, vol. 29, p. 237.

DEUEL, H. J., JR., E. MOVITT, L. F. HALLMAN AND F. MATTSON 1944 Studies of the comparative nutritive value of fats. I. Growth rate and efficiency of conversion of various diets to tissue. J. Nutrition, vol. 27, p. 107.

ERSHOFF, B. H., AND H. J. DEUEL, JR. 1944 Inadequacy of lactose and beta-lactose as dietary carbohydrates for the rat. J. Nutrition, vol. 28, p. 225.

FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL 1943 A report on margarine. Reprint and circular series, no. 118, 20 pp.

GEYER, R. P., R. K. BOUTWELL, C. A. ELVEHJEM AND E. B. HART 1943 Rations for the study of the relative nutritive value of fats and oils. Science, vol. 98, p. 499.

LINDQUIST, E. F. 1940 Statistical analysis in educational research. Houghton Mifflin, Boston, 264 pp.

WHITTIER, E. O., C. A. CARY AND N. R. ELLIS 1935 The effects of lactose on growth and longevity. J. Nutrition, vol. 9, p. 521.

ZIALCITA, L. P., JR., AND H. H. MITCHELL 1944 Corn oil and butterfat essentially equal in growth-promoting value. Science, vol. 100, p. 60.

THE INFLUENCE OF CHOLINE AND OF TRYPAN BLUE UPON THE UTILIZATION OF CAROTENE AND VITAMIN A FOR LIVER STORAGE OF VITAMIN A [1]

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ONE FIGURE

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According to Thorbjarnarson and Drummond ('38) the storage of fat or cholesterol in the liver tends to increase the total amount of vitamin A stored. When choline was included in the diet the amount of fat in the liver and the amount of vitamin A stored was decreased. In rats allowed to accumulate large reserves of vitamin A by dosing with a concentrate, and subsequently restricted to various modifications of a diet deficient in vitamin A, rapid loss of the vitamin from the liver was observed. The inclusion of a high proportion of fat appeared, however, to retard the rate of loss, this effect again being counteracted by the addition of choline. Very small amounts of vitamin were present in the livers of the rats in the series in which fatty livers were produced by high-fat and cholesterol-containing diets. In the series in which large doses of vitamin A were given, fatty livers were not produced except for mildly fat livers in one group. The data do not indicate that the rate of accumulation or depletion of liver vitamin A was dependent upon the level of fat in the liver although some relation to level of dietary fat was indicated.

[1] This study was supported by a grant from Swift and Co., Chicago.

Lease and Steenbock ('39) compared choline-low high-fat and low-fat diets containing adequate protein, and the same high-fat diet with and without choline, in similar depletion experiments. They found no differences in rate of vitamin A loss due to dietary or liver fat levels.

Popper and Chinn ('42) used the fluorescence microscope to study the distribution of the decreased amount of vitamin A which they found in the livers of choline-deficient rats fed carotene. Chemical analysis showed that these rats had from 1.3 to 19 I.U. vitamin A per gram of liver, in contrast with the normal value of more than 200 I.U. They stated that inability of the liver to convert carotene into vitamin A was not a major factor in causing this decrease since preliminary experiments had shown the same results when vitamin A replaced the carotene. No data on the vitamin A fed rats were given. Clayton and Baumann ('44) measured the depletion of vitamin A in the liver with and without choline using adult rats and mice which had initial uniform stores of vitamin A. They found no difference in the amount of hepatic vitamin A accumulated or retained on the high-fat diets regardless of their choline content or the amount of fat in the livers. In one series they used an adequate protein or methionine-low low-fat diet without choline upon which severe choline deficiency rapidly developed. In 10 or 20 days no difference in vitamin A content of livers, kidneys or other organs developed between the choline-fed and deficient groups. The fat content of the livers was not specified.

In view of these differences it was thought that possibly inability of the liver to convert carotene to vitamin A during choline deficiency might be a more important factor than Popper and Chinn suspected, since they found greater differences in the amount of vitamin A stored during choline deficiency than did the other workers who fed vitamin A rather than the provitamin. Furthermore, since the excess fat in the liver of rats on choline-deficient diets accumulates in the hepatic cells, if less vitamin A were stored by the choline-deficient rat than by the choline-fed rat receiving carotene

this might be indicative of the site of conversion of carotene to vitamin A.

Lasch and Roller ('36) in another attempt to discover the site of storage of vitamin A made a study of the role of the hepatic reticulo-endothelial system. This was done by injecting substances which were taken up by the reticulo-endothelial system in amounts just below the lethal dose, for 3 days prior to, and 3 days during, the injection of vitamin A.[2] Guinea pigs and rabbits whose reticulo-endothelial system had been blocked in this manner had a decreased storage capacity for vitamin A and in a few cases the animals' storage capacity was said to have been abolished completely. On the basis of the results reported the authors concluded that the storage of vitamin A in the liver depends on its reticulo-endothelial system (the Kupffer cells).

With these results in mind an attempt was made to determine the role of the Kupffer cells in the conversion of carotene to vitamin A by feeding carotene to rats whose reticulo-endothelial system had been blocked in a similar manner. Trypan blue was the only dye used in this study, and vitamin A storage was determined after the feeding of vitamin A or carotene.

Thus by packing the hepatic cells with fat by means of choline-deficient diets and the Kupffer cells with the dye, an effort was made to establish in one or the other the site of carotene transformation and of vitamin A storage.

METHODS

Chemical. The livers were analyzed for vitamin A by a modification of the Davies ('33) method, the Carr-Price blue color being read in the Evelyn photoelectric colorimeter. Total solids were determined by drying the samples in a vacuum oven at 50°C. Liver "fat," was determined by extracting the dried samples with anhydrous ethyl ether in a modified Soxhlet apparatus and weighing the "fat," which

[2] Vogan.

had been dried in a vacuum dessicator after evaporation of the ether.

THE CHOLINE EXPERIMENT

The low-fat diet. Rats from the stock colony were prepared as for a vitamin A assay, that is, the females were placed on a vitamin A-low skim milk diet for the last week of lactation. The weanling rats were continued on this diet to the twenty-eighth day at which time they had attained an average weight of 57 gm. They were then placed on the low-fat diet employed by Clayton and Baumann ('44) which contained hot-alcohol extracted casein 3.5, cystine 0.15, brewers yeast 3.5, salts [3] 2.9, cottonseed oil 1.5, and dextrin 88.5. Choline was added as 0.3% of the diet fed the control groups. The animals received the experimental diet, which was vitamin A-free, for 1 week without any additional vitamin A, after which the vitamin A [4] or carotene [5] oil supplement was given for 1 month at which time they were sacrificed. The oil supplement was given three times weekly by stomach tube and the other vitamin supplements were injected six times weekly, at a level of 20 μg daily of thiamine, riboflavin and pyridoxine, and 100 μg daily of Ca pantothenate and nicotinic acid amide. The B vitamins were given to supplement the somewhat inadequate amount of yeast in the diet. The litters were evenly divided into groups of ten or more rats of each sex. One group of male and one group of females rats fed carotene also received 3 mg mixed tocopherols [6] daily. The livers from each group of rats were pooled for chemical analysis. Some rats from each group were sacrificed in order to confirm the absence of vitamin A in the liver before the oil supplements were given.

The high-fat diets. Similarly prepared rats were placed on a diet containing hot-alcohol-extracted casein 10, fat 35,

[3] Hubbell, Mendel and Wakeman '37.

[4] The vitamin A was given as reference cod liver oil, 0.42 gm total per week.

[5] Carotene in oil concentrate, 2.2 mg per gram. The total amount of oil given was 0.8 gm per week.

[6] Concentrate of natural mixed tocopherols, 40%. This was kindly supplied by K. Hickman of Distillation Products, Inc., Rochester, N. Y.

sucrose 51, and salts [3] 4, when the males weighed approximately 98 and the females 88 gm. The B vitamin supplements were given as in the low-fat diet. Choline was again added as 0.3% of the diet for half the groups. The same oil supplements used with the low-fat diet were given after the animals had been on the high-fat diets for 10 to 12 days. The groups which were to receive carotene plus tocopherols were omitted and the diet was prepared with two different types of fat instead, lard and hydrogenated cottonseed oil. Since lard, commonly used in high fat diets, is known to be deficient in vitamin E, and is highly oxidative, the hydrogenated cottonseed oil which contains tocopherols was employed for half of the animals.

Preliminary vitamin A analysis of livers. Seven rats died as a result of choline injection when they were first placed on the low-fat diet. The injection of choline was then discontinued and it was incorporated in the diet. The pooled livers of these weanling rats were found to contain 2.6 μg vitamin A per gram. The livers of twelve rats sacrificed after 1 week on the low-fat diet without any vitamin A supplement were tested individually for vitamin A and all were found to be negative. One rat that received carotene and tocopherols in addition to the choline-deficient diet, killed on the second day was found to have 7 μg vitamin A in its liver. Two similarly treated rats had 6 and 13 μg vitamin A per liver after 20 days of supplementary feeding, while a rat that received choline and vitamin A for the same period had 44 μg of the vitamin in its liver. The livers of three rats receiving the high-fat diet for 1 week without the vitamin were found to be devoid of vitamin A.

RESULTS

Changes in body weight. The rats on the low-fat diet deficient in choline gained twice as much weight as did those which received choline. The reverse was true of the groups fed the high-fat diets. Growth on none of these diets was

normal either with or without choline, but the high-fat diets were superior to the low-fat diet.

Mortality. All rats on the low-fat diet survived with the exception of the animals accidentally killed. There were no accidental casualties among the animals on the high-fat diets, but three rats on each type of fat-diet deficient in choline died before the termination of the experiment.

The fat content of the livers. When choline was withdrawn from the diet fatty livers resulted in all cases, the effect being greatest on the lard diet and least on the low-fat diet. As the fat content and total weight of the livers increased, the moisture content decreased. When choline was given the total weight, fat and moisture of the livers were normal.

Vitamin A storage in the liver. Interpretation of the data involved comparisons of amounts of vitamin A per gram of fresh liver tissue, per gram of non-fat fresh liver, per gram of non-fat liver solids, per gram of liver fat, and per liver. The ability of the active liver tissue to accumulate the vitamin was probably best represented by the vitamin concentration of the non-fat liver solids, or by the total amount of the vitamin retained per liver, since these measures eliminate the influence of the liver fat upon the apparent vitamin concentration. The data are presented in table 1 and the total vitamin A per liver and the concentration of fat in the livers compared in figure 1. Since sex and the addition of tocopherols in some of the carotene groups did not influence the results significantly, all groups on the same diets and receiving the same vitamin supplement were combined in the figure.

On all diets very low stores were produced on 250 μg carotene daily, 50 μg per liver being the maximum. On the low-fat diet with choline this storage amounted to about one-third of that acquired from 100 I.U. vitamin A daily; without choline it approximated that produced by the vitamin A dosage. On the high-fat diets liver vitamin A from carotene feeding was less than a tenth of the amount produced by vitamin A feeding and was practically negligible.

TABLE 1

The storage of vitamin A in 28 days as influenced by the addition of choline to low-fat and high-fat diets.

DIET	SOURCE OF VITAMIN PER DAY	SEX	NUMBER OF ANIMALS	AVERAGE WEIGHT GAIN	LIVER			LIVER VITAMIN A			
					Weight	Fat	Solids	Per gm	Per liver	Per gm non-fat solids	Per gm fat-free liver
				gm	gm	%	%	μg	μg	μg	μg
Low- fat with choline	100 I.U. vitamin A	♂	9	2	2.9	4.1	29	32	93	128	33
		♀	9	8	2.8	3.9	29	68	190	272	71
	250 μg carotene	♂	8	4	2.8	3.7	29	13	36	52	14
		♀	9	3	2.8	3.7	30	18	50	69	19
	250 μg carotene with tocopherols	♂	6	9	3.3	3.8	30	8	26	31	8
		♀	11	5	2.7	4.2	31	16	43	59	17
Low-fat without choline	100 I.U. vitamin A	♂	12	10	3.3	18.1	39	12	40	57	14
		♀	11	6	2.9	20.7	41	23	67	115	30
	250 μg carotene	♂	11	14	3.0	14.0	37	13	39	56	15
		♀	7	14	3.0	13.8	38	7	21	29	8
	250 μg carotene with tocopherols	♂	10	18	3.6	11.4	39	7	25	25	8
		♀	9	15	3.2	13.2	38	10	32	40	11
Lard with choline	100 I.U. vitamin A	♂	7	35	6.0	3.8	30	28	168	108	29
		♀	6	32	4.8	3.7	30	32	153	123	33
	250 μg carotene	♂	8	31	5.3	4.7	30	2	10	8	2
		♀	7	26	4.6	3.5	30	3	13	11	3
Lard without choline	100 I.U. vitamin A	♂	8	17	11.1	34.4	48	13	145	95	20
		♀	7	16	8.7	44.1	57	21	182	154	38
	250 μg carotene	♂	8	22	10.8	36.8	51	0	0	0	0
		♀	6	20	9.4	39.1	54	0	0	0	0
Hydrogenated cottonseed oil with choline	100 I.U. vitamin A	♂	8	29	5.6	3.0	30	39	218	144	40
		♀	6	26	4.3	2.9	29	52	223	200	54
	250 μg carotene	♂	8	35	6.1	2.4	29	2	12	8	2
		♀	6	26	4.5	3.1	29	6	27	23	6
Hydrogenated cottonseed oil without choline	100 I.U. vitamin A	♂	6	7	8.9	34.1	49	27	240	180	40
		♀	6	11	8.5	32.4	49	33	280	250	49
	250 μg carotene	♂	6	22	9.8	33.6	50	1	10	6	1
		♀	6	7	8.7	33.6	50	1	9	6	1

On the low-fat diet. On the low-fat diet where fat deposition was relatively low, when choline was withheld the rats receiving vitamin A stored less than one-half as much as did those which received choline, compared on any of the bases available (table 1). When carotene was given, the amount of vitamin A stored was not influenced by the withdrawal of choline. There was in the latter case, some reduction in the amount of vitamin A stored by the female animals but

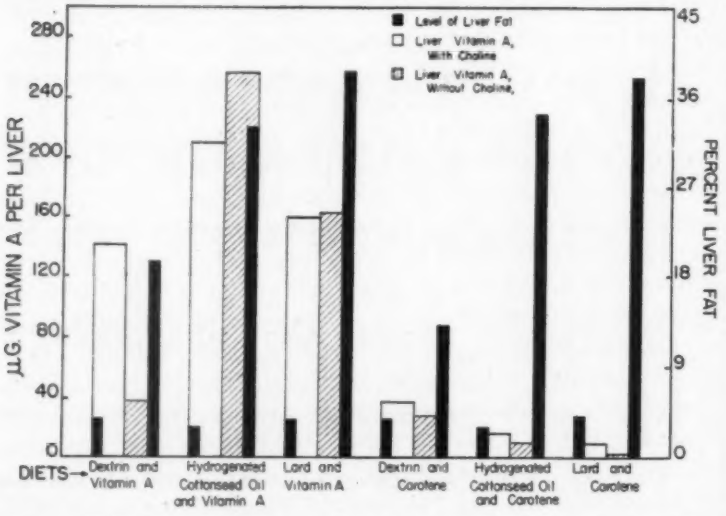

Fig. 1 Total vitamin A and per cent fat in the livers of rats fed dextrin (low fat) and high fat diets with and without choline.

this was not considered to be significant. The amount of vitamin A stored was not influenced by feeding tocopherols with the carotene in this experiment.

On the high fat diets. On the lard diet where fat deposition was the greatest, the total amount of vitamin A stored in the livers, as well as the concentration per gram of fat-free liver or liver solids, was nearly the same with and without choline-when vitamin A was fed. The concentration of the vitamin in the fresh liver tissue was reduced in the latter case due to

the increased size of the fatty livers (table 1). This was also true on the hydrogenated cottonseed oil diet but in this case the withdrawal of choline resulted in a small increase of doubtful significance in the total amount of vitamin A stored. The accumulation of the vitamin appeared to be consistently greater on the cottonseed oil than on the lard.

When carotene was fed the animals on the high-fat diets stored less vitamin A than did those on the low-fat diet. The amount of storage was less when choline was withdrawn from these diets but it is not likely that this lowering was significant at these extremely low levels.

Sex. There seemed to be no clearly established pattern of response which could be attributed to a difference in sex, although in most of the groups the females stored more vitamin A than did the males.

CHOLINE EXPERIMENT

Discussion

Vitamin A. The increase in the fat content of the liver induced by a dietary deficiency of choline was associated with a decrease in the vitamin A deposit in the liver of rats receiving a fixed amount of vitamin A with the low-protein, low-fat, high carbohydrate diet. On the low-protein, high-fat diets no such effect was seen. Liver fat deposition was least on the low-fat choline-free diet, hence if vitamin A storage were inversely related to this factor the least effect of the choline should have occurred in this case. But definite improvement in storage with choline was seen only on this diet.

Carotene. Differences in deposition of vitamin A in the liver due to choline feeding were small on both types of diet when carotene was fed. In general there was less vitamin A in the liver when choline was absent from the diet but the differences were so small as to be of doubtful significance. In this experiment carotene utilization was inferior on the high fat diets. The large amount of fat in the diet may have reduced the absorption of carotene or the greater weight gains

of these groups may have involved more utilization of the vitamin. However, a similar difference due to growth in the stores of vitamin A fed as such was not observed.

Lease and Steenbock ('39), Clayton and Baumann ('44) and Thorbjarnarson and Drummond ('38) noted the rate of depletion of vitamin A stores on high and low-fat diets or in choline deficiency. In this study the accumulation of such stores on uniform intake was observed.

Lease and Steenbock noted no effect on depletion on choline-low high-fat and low-fat diets nor on high-fat diets with and without choline. Our findings are similar. The effects of low-fat diets with and without choline were not compared by Lease and Steenbock. Clayton and Baumann used both high-fat and low-fat diets with and without choline and detected no difference in rate of depletion of liver vitamin A. In one series of rats on low-methionine low-fat diet for 10 or 20 days substantially the same vitamin A retention appeared to have occurred with and without choline. This is not in accord with our results, but the basal diet used was different from any which we employed. Our findings accord however with the observation of Thorbjarnarson and Drummond that high-fat diets retarded the loss of liver vitamin A, but not in proportion to the amount of fat in the livers. The marked influence of choline on carotene utilization noted by Popper and Chinn ('42) was not seen in this experiment and this lack of confirmation may have been due to the type of diet employed by them. It appears that the character of the basal diet is more important in the accumulation of hepatic vitamin A than the presence or absence of choline or fatty livers. This may also be true of the depletion of these stores.

Apparently whatever may be the mechanisms controlling the utilization of vitamin A and carotene, they are distinct and are not affected by the same condition.

THE TRYPAN BLUE EXPERIMENT

The male rats from the litters of four stock-colony rats were carried to weaning in the manner described for the

choline experiments. They were then weaned at 21 days of age directly to a vitamin A-deficient diet made up of casein (alcohol extracted) 22 parts, hydrogenated cottonseed oil 5, brewery yeast 10, cornstarch 60.5, and salts [3] 2.5. The animals were allowed to continue on this diet until their weight curves began to plateau, indicating the onset of vitamin A deficiency. Trypan blue in 1% solution was injected for at least 2 days before any vitamin feeding, and by this time the dye had turned the skin a bright blue. The original plan was to inject 0.5 ml for 5 days followed by a 2-day rest period. Due to evidence of toxicity the number of injections was reduced to 3 in the second and third week, but the full five injections were given during the first and final weeks. Comparable groups of animals selected from the same litters were injected with distilled water. In each case half of the animals received 0.5 mg carotene daily in 0.23 gm oil and half received 60 I.U. vitamin A daily as 0.036 gm reference (II) cod liver oil. The oil supplements were given 6 hours after the injection of the dye and none was given during the 2-day rest period. After receiving this treatment for a period of 4 weeks the animals were sacrificed and the individual livers were analyzed chemically for vitamin A.

Results

There was no decrease in the amount of vitamin A stored by the dye-treated rats when the vitamin itself was fed, although the growth was depressed. Two of the animals had more liver-vitamin A than did animals which had been injected with water, (table 2). When carotene was fed the animals surviving the injection showed depressed growth and somewhat smaller liver stores than their water-injected littermates.

Where the animal failed to survive the trypan blue injections the liver stores were lower regardless of whether the vitamin or the pro-vitamin had been given. This may have been due either to the shorter period of vitamin feeding or to

TABLE 2

The effect of trypan blue injections on rats depleted of vitamin A and then fed carotene or vitamin A.

RAT NUMBER	TRYPAN BLUE, 1% SOL. INJECTED	SUPPLEMENT GIVEN		PERIOD OF VITAMIN FEEDING	LIVER VITAMIN A		GROWTH				FINAL WEIGHT	DEFICIENCY SYMPTOMS
							Weight gain per week					
		Vitamin A	Carotene		Per gm	Total	1st	2nd	3rd	4th		
	ml	*I.U.*	*mg*	*days*	*γ*	*γ*	*gm*	*gm*	*gm*	*gm*	*gm*	
17	0.5	1680		28	3.7	44	4	0	35	33	194	
19	0.5	1680		28	3.5	36	− 3	9	24	39	173	+
23	0.5	1680		28	trace	trace	12	− 24	25	6	122	++
28	0.5	1680		28	2.5	60	9	2	30	28	160	+
27	0.5	1440		24[1]	trace	trace	19	15	41	..	203	+
13	0.5	540		9[1]	trace	trace	− 4	..	..	..	86	+
				average	1.6	23						
14	..	1680		28	2.9	32	32	38	37	4	236	
20	..	1680		28	4.5	40	29	32	38	− 4	192	
25	..	1680		28	1.6	21	43	48	41	31	286	
29	..	1680		28	2.6	27	33	38	35	20	227	
				average	2.9	29						
15	0.5		14.0	28	21	181	24	− 6	6	24	170	+
21	0.5		14.0	28	13	105	15	10	− 3	21	152	+
24	0.5		14.0	28	18	171	− 2	8	1	30	154	+
30	0.5		5.5	11[1]	10	70	12	..	..	..	133	
16	0.5		4.5	9[1]	8.9	40	− 4	..	..	..	92	
31	0.5		4.5	9[1]	5.6	35	1	..	..	..	99	
				average	12.8	100						
22	..		14.0	28	23	203	19	18	24	27	205	
18	..		14.0	28	22	243	23	19	28	32	226	
26	..		14.0	28	18	184	32	22	36	22	232	
32	..		14.0	28	21	177	22	32	21	21	196	
33	..		14.0	28	17	170	32	23	28	22	225	
				average	20	195						

[1] The animal died before the termination of the experiment.

the occurrence of an initial interference with storage followed by the setting up of a compensatory mechanism. The latter possibility is further indicated since during the early part of the supplementary feeding the injected rats, particularly those receiving vitamin A, failed to gain and several developed symptoms of the deficiency.

Discussion

The decreased storage of vitamin A observed by Lasch and Roller ('36) in guinea pigs and rabbits was not observed in this experiment with rats when the animals were injected with trypan blue. The total storage of vitamin A by the dye-injected rats that had been fed carotene was lower than that seen in the water-injected animals. It is doubtful, however, whether this can be considered significant inhibition of carotene conversion. Examination of the livers of animals sacrificed during the first and second week of dye injection might have indicated more definite differences. There is some indication of an "all or none" phenomenon in the rats fed vitamin A, since the stores of the vitamin in the livers of the dye-treated animals were either normal or negligible. The carotene feeding produced more graduated differences. This may point to different mechanisms for transformation and storage in the liver.

SUMMARY

The deposition of vitamin A in the livers of depleted rats fed fixed amounts of vitamin A along with high-fat low protein diets was not affected by the presence of excess liver fat or choline deficiency. With a low-fat low-protein basal diet the addition of choline increased the liver vitamin A.

When carotene was fed with the high-fat diets the liver vitamin deposits were very small and were somewhat less in the fatty than in the normal livers. With the low-fat diet the carotene produced better vitamin deposition, which was little affected by the presence or absence of choline.

The hepatic accumulation of vitamin A was much the same when the vitamin was fed with the high-fat and low-fat diets, regardless of liver fat, except for a slight decrease on the lard diet. When carotene was fed there was a definite depression of vitamin A storage on the high-fat as compared with the low-fat diets.

The injection of trypan blue produced no effect upon the deposition of vitamin A in the livers of rats fed a fixed amount of the vitamin. A depression of doubtful significance occurred in the deposits found in similar animals fed relatively large amounts of carotene.

It is concluded that the mechanisms of utilization and storage of vitamin A and carotene are probably affected by different conditions and that the composition of the accompanying vitamin-deficient basal diets is an important factor in determining their efficiency.

LITERATURE CITED

CLAYTON, C. C., AND C. A. BAUMANN 1944 Vitamin A storage and factors that affect the liver. J. Nutrition, vol. 27, p. 155.

DAVIES, A. W. 1933 Colorimetric determination of vitamin A by the alkali digestion method. Biochem. J., vol. 27, p. 1770.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

LASCH, F., AND D. ROLLER 1936 Uber die Beeinflussung des Vitamin A-Stoffwechsels der Leber durch Blockade des Recticulo-endothels. Klin. Woch., vol. 15, p. 1636.

LEASE, E. J., AND H. STEENBOCK 1939 Diet and rate of depletion of hepatic vitamin A. J. Nutrition, vol. 17, p. 85.

POPPER, H., AND H. CHINN 1942 Changes of vitamin A distribution in choline deficiency. Proc. Soc. Exp. Biol. and Med., vol. 49, p. 202.

THORBJARNARSON, T., AND J. C. DRUMMOND 1938 Conditions influencing the storage of vitamin A in the liver. Biochem. J., vol. 32, p. 5.

THE NUTRITIVE VALUE OF CANNED FOODS

XVII. PYRIDOXINE, BIOTIN AND "FOLIC ACID" [1]

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Since the National Canner's Association-Can Manufacturer's Institute Nutrition Program was inaugurated 3 years ago, a great many values for the vitamin A, carotene, thiamine, niacin, riboflavin, pantothenic acid, and ascorbic acid content of canned foods have been obtained (Ives et al., '44, '45; Pressley et al., '44; Thompson et al., '44). After study of the older, better-known vitamins had been completed, it was decided to conduct a survey of canned foods to determine the content of some of the newer B-complex factors: pyridoxine, biotin and "folic acid."

EXPERIMENTAL PROCEDURE

The method of collection of the samples was essentially the same as that described by Clifcorn ('44). Approximately ten samples of each of the following products were assayed for the factors under consideration: green asparagus, carrots, green beans, yellow corn, grapefruit juice, peaches, peas, salmon, spinach and tomatoes. The samples were assayed within 6 weeks of the time of their arrival and were stored at room temperature during that period.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Now at Research Department, American Can Company, Maywood, Illinois.

Preparation of samples

A sample for analysis consisted of six consumer size cans or one to three no. 10 cans. The solids and liquids were separated by the use of a drained solids screen. The solids were weighed, and the liquid volume determined. In order to determine the distribution of nutrients between the solid and liquid portions of the can, the solids and liquid portion of five samples of each product were analyzed separately. In these instances 100 gm aliquots of the combined solids of a sample were blended in a Waring blendor with 100 ml of water and 2 to 3 ml of chloroform. Chloroform was added directly to the 100 ml aliquots of the combined liquids and to all of the grapefruit juice samples. For the five remaining samples of each product, one-tenth of the solid and liquid portion samples were recombined and this reconstituted aliquot blended with 2 to 3 ml of chloroform. The brine was discarded from all of the salmon samples, and they were treated in the same manner as the solid aliquots.

Assay methods

Pyridoxine. Samples were prepared and assayed according to the procedure of Atkins et al. ('43). Saccharomyces carlsbergensis, the organism employed, responds approximately equally to pyridoxine, pyridoxamine, and pyridoxal (Snell and Rannefeld, '45). Duplicate assays were set up on different days.

Biotin. The samples were assayed according to the procedure of Shull, Hutchings and Peterson ('42) using the modification of the medium reported by Shull and Peterson ('43). The samples were hydrolyzed for assay by autoclaving with 4 N sulfuric acid for 2 hours at 15 pounds pressure.

"Folic acid." The samples were analyzed for both the S. lactis activity and the L. casei activity against a standard of crystalline vitamin B_c. The S. lactis factor was measured by the method of Luckey, Briggs and Elvehjem ('44). The titrimetric method was employed which necessitated the inclusion of 200 mg of sodium citrate per 10 ml of the medium.

Thymol blue was the indicator used. The L. casei factor was measured by the method of Teply and Elvehjem ('45).

Samples were prepared for both assays in the same way by incubation with takadiastase (0.1 gm of enzyme per 5 gm of sample) at 37°C. for 24 hours under toluene. The digests were then neutralized and autoclaved at 15 points pressure for 15 minutes (Luckey, Briggs, Moore, Hart and Elvehjem, '45; and University of Texas Publication no. 4237, '42).

RESULTS AND DISCUSSION

The results of this survey are summarized in table 1. The values reported are in terms of micrograms of the vitamin per 100 gm of the entire, original contents of the can except in the case of salmon where the brine was discarded.

The pyridoxine values were considerably higher than those for biotin and folic acid. The range for pyridoxine in individual samples of any product was three-fold or less in all instances with the exception of grapefruit juice and salmon where it was four-fold. Salmon contained two or three times as much pyridoxine as the vegetables and almost ten times as much as the fruit.

Fruit contained the smallest amount of biotin while the fish product contained the largest. The range of this factor was observed to be rather small, being three-fold or less in all of the foods analyzed except peaches.

The S. lactis factor of the folic acid complex showed the same range of values as biotin while the values for S. lactis factor averaged 30 to 50% of the L. casei factor content. However, both of them were present in much greater amounts in spinach and green asparagus, and in lower amounts in salmon than was biotin. In green asparagus, yellow corn, grapefruit juice, peas and salmon the range was two-fold or less for the L. casei factor while this small range occurred only in green asparagus and yellow corn samples for the S. lactis factor. The greatest range for the L. casei factor was four-fold in spinach while the greatest ranges for the S. lactis factor were seven-fold in green beans and four-fold in salmon and spinach.

TABLE 1

Pyridoxine, biotin, S. lactis factor and L. casei factor content of canned foods on the fresh weight basis.

PRODUCT	NO. OF SAMPLES	PYRIDOXINE		BIOTIN		S. LACTIS		L. CASEI	
		Range	Ave.	Range	Ave.	Range	Ave.	Range	Ave.
		μg/100 gm		*μg/100 gm*		*μg/100 gm*		*μg/100 gm*	
Asparagus, green	10	17– 48	30	1.0 – 2.1	1.7	3.2– 7.9	5.8	6.2–11.2	9.0
Beans, green	11	18– 52	32	1.1 – 1.8	1.3	1.2– 8.3	2.9	4.0–11.0	7.7
Carrots	10	14– 30	22	1.0 – 2.0	1.5	1.0– 2.2	1.3	2.2– 5.7	4.1
Corn, yellow	10	41–100	68	1.4 – 2.7	2.2	1.3– 2.4	1.7	3.3– 6.9	5.6
Grapefruit juice	11	7– 27	14	0.3 – 0.4	0.3	0.3– 0.7	0.5	1.0– 1.6	1.2
Peaches	9	11– 20	16	0.05– 0.3	0.2	0.3– 1.2	0.5	1.0– 2.9	1.5
Peas	10	24– 72	46	1.4 – 3.5	2.1	1.0– 2.2	1.7	3.2– 5.2	4.4
Salmon	10	70–260	130	6.7 –15.2	9.8	1.0– 4.4	2.6	5.3–10.2	6.9
Spinach	10	45– 80	60	1.1 – 3.1	2.3	4.0–16.5	7.4	11 –51	20.7
Tomatoes	10	47– 98	71	0.8 – 2.4	1.8	1.5– 4.1	2.7	3.9– 8.2	5.4

The ranges of the other products were three-fold or less for both factors.

The per cent distribution of these vitamins between the solids and the liquid of the can is shown in table 2. Pyridoxine distribution was approximately 60% in the solids and 34% in the liquid. This distribution is essentially the same as that found for thiamine and riboflavin by Brush et al. ('44). Seventy-seven to 99% of the biotin was found in the drained solids in all products analyzed except tomatoes. Due to the

TABLE 2

The distribution of pyridoxine, biotin, S. lactis factor, and L. casei factor between the solids and liquids of canned foods.

PRODUCT	WEIGHT	PYRIDOXINE	BIOTIN	S. LACTIS FACTOR	L. CASEI FACTOR
		% of total present in the solids[1]			
Asparagus, green[2]	62	65	91	68	65
Beans, green	60	63	85	67	64
Carrots	67	66	77	74	72
Corn, yellow	69	72	88	77	73
Peaches	65	67	89	80	78
Peas	63	64	88	83	66
Spinach	62	64	99	67	63
Tomatoes	57	66	68	63	66

[1] Average values for 5 different samples of each product obtained by separate analysis of the liquids and drained solids. The difference between the figures given and 100 represents the percentage found to be present in the liquid in each instance.

[2] Six samples analyzed.

high moisture content and fragile structure of the canned tomatoes, the separation of the drained solids was not as distinct on the drained weight screen as was the case with the other products. Biotin in the bound state is the least water soluble of any of the B-complex. Seventy-two to 80% of both the S. lactis and L. casei factors occurred in the solids of carrots, yellow corn, and peaches while the distribution was about two-thirds in the solids and one-third in the liquids in the other products. In general, except for biotin, there was rather close agreement between the percentages of the total weight contributed by the solids in the can and the percentages of these vitamins contained in the solids.

SUMMARY

Pyridoxine, biotin and "folic acid" values on 101 samples of canned foods are reported in this paper. The distribution of these factors between the solids and the liquids of the product is also reported. Salmon, yellow corn, and tomatoes are good sources of pyridoxine. Approximately two-thirds of this vitamin was found in the solids of the product. Salmon had the highest biotin content. Sixty-eight to 99% of the biotin was present in the solids of the products analyzed. "Folic acid" measured as L. casei and S. lactis activity against crystalline vitamin B_c was found in the highest quantity in green vegetables where about 64% of the factors occurred in the solids in each instance.

LITERATURE CITED

ATKIN, LAWRENCE, ALFRED S. SCHULTZ, WILLIAM L. WILLIAMS AND CHARLES N. FREY 1943 Yeast microbiological method for the determination of pyridoxine. Ind. and Eng. Chem., Anal. Ed., vol. 15, p. 141.

BRUSH, MIRIAM K., WINIFRED F. HINMAN AND EVELYN G. HALLIDAY 1944 Nutritive value of canned foods. V. Distribution of the water-soluble vitamins between solid and liquid portions of canned vegetables and fruit. J. Nutrition, vol. 28, p. 131.

CLIFCORN, L. E. 1944 Nutritive value of canned foods. J. Nutrition, vol. 28, p. 101.

IVES, MARGARET, J. R. WAGNER, C. A. ELVEHJEM AND F. M. STRONG 1944 Nutritive value of canned foods. III. Thiamine and niacin. J. Nutrition, vol. 28, p. 117.

IVES, MARGARET, MARIE ZEPPLIN, S. R. AMES, C. A. ELVEHJEM AND F. M. STRONG 1945 Nutritive value of canned foods. VIII. Riboflavin, niacin and pantothenic acid. J. Am. Dietetic Assn., vol. 21, p. 357.

LUCKEY, T. D., G. M. BRIGGS, JR. AND C. A. ELVEHJEM 1944 The use of Streptococcus lactis R for the measurement of "folic acid." J. Biol. Chem., vol. 152, p. 157.

LUCKEY, T. D., G. M. BRIGGS, JR., P. R. MOORE, E. B. HART AND C. A. ELVEHJEM 1945 Studies on the liberation of compounds in the folic acid group. J. Biol. Chem., vol. 161, p. 395.

PRESSLEY, ANNE, CLARE RIDDER, M. C. SMITH AND EMILY CALDWELL 1944 Nutritive value of canned foods. II. Ascorbic acid and carotene or vitamin A. J. Nutrition, vol. 28, p. 107.

SHULL, G. M., B. L. HUTCHINGS AND W. H. PETERSON 1942 A microbiological assay for biotin. J. Biol. Chem., vol. 142, p. 913.

SHULL, G. M., AND W. H. PETERSON 1943 Improvements in the Lactobacillus casei method for biotin. J. Biol. Chem., vol. 151, p. 201.

SNELL, ESMOND E., AND A. N. RANNEFELD 1945 The vitamin B_s group III. J. Biol. Chem., vol. 157, p. 475.

TEPLEY, L. J., AND C. A. ELVEHJEM 1945 The titrimetric determination of the "Lactobacillus casei factor" and "folic acid." J. Biol. Chem., vol. 157, p. 303.

THOMPSON, MARY LOUISE, ELIZABETH CUNNINGHAM AND ESMOND E. SNELL 1944 The nutritive value of canned foods. IV. Riboflavin and pantothenic acid. J. Nutrition, vol. 28, p. 123.

UNIVERSITY OF TEXAS PUBLICATION 1942 The vitamin content of tissues. II. No. 4237.

NUTRITIONAL STATUS OF RATS ON MILK DIETS CONTAINING SUCCINYLSULFATHIAZOLE [1]

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Feeding experiments with mineralized cows' milk have shown that a good nutritional status can be maintained in rats by such a diet (Underhill, Orten, Mugrage and Lewis, '33). Although some evidence suggests that milk is a good source of all the B vitamins, there is proof to the contrary (Williams, Cheldelin and Mitchell, '42; Day, Wakim, Zimmerman and McClung, '44). Furthermore, available data show that cows' milk is generally a poor source of vitamin K and that at times it may be almost entirely devoid of activity (Sells, Walker and Owen, '41; Mueller and Wertz, '45). Therefore, it is possible that a milk diet supports an intestinal flora favorable for the production of adequate amounts of such needed factors. Accordingly, this investigation was planned to determine the nutritional adequacy of milk for rats deprived of vitamins which are known to be synthesized by the normal intestinal flora. An attempt was made to prevent vitamin synthesis by a combination of cecectomy and succinylsulfathiazole (SST) feeding, but because the animals developed a severe diarrhea, this was abandoned. Therefore, only SST was used to inhibit vitamin synthesis.

[1] Supported in part by a grant from the Research Fund of the Graduate School of Indiana University.

EXPERIMENTAL

Weanling piebald rats from our stock colony were used and were divided as equally as possible on the basis of litter membership, sex and weight. Most of the rats were housed in individual cages with screen floors, but a few were kept in screen-bottom group cages with 3 or 4 animals per cage.

Evaporated milk[2] and powdered whole milk,[3] purchased on the open market, were used. To mineralize the powdered milk, 1.2 gm of ferric citrate, 200 mg of manganous chloride, and 10 mg of copper sulfate were mixed with 1000 gm of the milk powder. Rats given evaporated milk were supplemented daily except Sundays, with 0.2 mg of copper, 3.4 mg of manganese and 2.4 mg of ferrous iron, each as the sulfate, in concentrated aqueous solution. The solutions were pipetted into small amounts of milk in food cups. Later in the day milk was allowed ad libitum. Drinking water was available to all animals.

The insoluble sulfonamide was administered as the sodium salt in the experiments involving evaporated milk. This was prepared by suspending 40 gm of SST in 165 ml of water and then adding 15 gm of sodium bicarbonate. As soon as the SST was completely dissolved and carbon dioxide ceased to evolve, the clear solution was stored in a brown bottle. Each day the required amount of SST solution was added to milk from freshly opened cans and mixed with it by vigorous stirring. The milk for control animals given no SST was treated similarly by adding equivalent amounts of sodium bicarbonate in solution.

RESULTS

The effects on growth of rats given SST in amounts ranging from 0 to 10% of the total milk solids fed are summarized in table 1. Growth impairment occurred at SST levels as low as 4%. The effect was noticeable from the second week

[2] Wilson's irradiated evaporated milk (Indianapolis, Indiana).

[3] The powdered whole milk was purchased from the Hoosier Condensed Milk Company, Bluffton, Indiana.

and became progressively more pronounced with time. After 6 to 9 weeks, at the higher levels of SST, growth became almost stationary in most of the animals. However, a small percentage increased in weight almost as rapidly as the controls which received no SST. A few animals died after 10 to 14 weeks. The few rats which developed severe urinary tract concretions suddenly refused food and died within a few days. In others which gradually declined and died, gross examination revealed no evidences of such concretions.

TABLE 1

Effect of succinylsulfathiazole on the growth of rats fed mineralized milk.

NO. OF RATS	SEX	LEVEL OF SST IN DIET [1]	AVERAGE WEEKLY WEIGHT						
			0	2	4	6	8	10	12
			gm	gm	gm	gm	gm	gm	gm
Evaporated milk									
16	M	10	48	78	103	123	129	138	140
21	M	5	50	89	122	146	174	176	167
9	M	0	40	80	131	185	225		
20	F	10	51	75	93	104	110	121	119
21	F	5	42	71	99	118	130	128	
8	F	0	36	76	108	137	155	175	185
Dried milk									
5	M	8	52	103	141	157	153	156	
9	M	4	52	110	175	224	253	263	277
9	M	0	49	117	185	251	282	315	326
4	F	8	47	89	113	120	124		

[1] Grams per 100 gm of milk solids.

Observations were made on the effects of "folic acid" and a liver concentrate on the growth of rats fed SST in dried or evaporated milk. "Folic acid" was prepared for administration, by dissolving the powder in water in such amounts that the solution contained 90 μg per milliliter. In most cases 18 μg were given per rat daily for a minimum of 3 weeks after the animals had been on the experimental diets 4 to 10 weeks. Larger amounts of "folic acid" seemed to have no greater effect on the growth or appearance of the rats. The

liver concentrate was prepared for administration as an aqueous solution contining 0.4 gm per milliliter. The dosage was 0.2 gm per rat daily.

The results summarized in table 2 clearly show a definite growth response in SST-fed rats given "folic acid" or liver. Animals given the larger amounts of SST showed a greater growth response upon administration of either supplement. No improvement in growth occurred in the SST-fed animals given a mixture of thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and inositol. Likewise, feeding xanthopterin [4] did not improve the growth or appearance of the animals.

In some experiments the liver fraction was fed continuously for 10 to 12 weeks after restriction to the milk-SST diets. Under these conditions the growth and appearance of the animals was essentially the same as in controls not given SST. Furthermore, rats on the SST-free evaporated milk diets grew as rapidly as similar animals given liver concentrate regularly for the first 8 weeks. This suggests that liver concentrate furnishes nothing essential to the growth of milk-fed rats if synthesis of vitamins by intestinal microorganisms is unimpaired.

Prothrombin time determinations were made on diluted plasma (12.5%) from more than 100 rats given evaporated milk, and on 30 that were fed the dried milk. Changes from normal were found only in rats fed the SST at a level of 10%. Whereas none of the animals receiving less than 10% SST had prothrombin time values over 51 sec., the values were above 65 in a number of the 10% SST group, but many were in the normal range (40 ± 2.2 sec.) The plasma prothrombin time returned to the normal range following the administration of 2-methyl-l, 4-naphthoquinone.

Bacterial counts were made on the cecal contents of rats that had been on the different diets for 70 to 150 days. Appropriate dilutions were prepared in sterile tap water and

[4] The xanthopterin was synthesized by Drs. P. H. Hidy and F. W. Neumann, using the Koschara ('43) method.

TABLE 2

Effect of supplements on the growth of rats fed mineralized milk plus succinylsulfathiazole.

NO. OF RATS AND SEX	LEVEL OF SST IN DIET [1]	SUPPLEMENT ADDED	DAYS ON DIET BEFORE ADDING SUPPL.	AVERAGE GAIN BEFORE AND AFTER ADDING THE SUPPLEMENT					
				Days before [2]			Days after		
				21	14	7	7	14	21
				gm	*gm*	*gm*	*gm*	*gm*	*gm*
			Evaporated milk						
10(5M)	10	"Folic acid"	56	10	2	−3	11	26	43
9(4M)	10	None	54	16	9	5	3	4	7
4(1M)	10	Xanthopterin [3]	57	−1	2	3	−12	−24	
3(0M)	10	None	57	0	7	1	−7	−16	
6(6M)	5	"Folic acid"	69	21	8	2	23	39	53
6(4M)	5	"Folic acid"	52	29	14	0	9	28	44
3(3M)	5	None	49	25	12	9	5	14	13
3(3M)	0	None	47	51	29	17	22	49	57
3(0M)	5	Vitamin mixt. [4]	74	0	7	2	5	6	
3(0M)	5	None	74	15	9	3	8	18	
2(2M)	0	"Folic acid"	35	45	26	8	23	55	82
2(2M)	0	None	35	63	28	22	33	58	99
5(1M)	0	Liver extract	52	32	19	3	13	24	41
5(1M)	0	None	52	41	20	15	17	28	42
			Dried milk						
4(2M)	8	"Folic acid"	54	10	10	5	22	32	
9(4M)	8	Liver extract	31	51	21	11	14	30	43
8(4M)	8	None	32	53	22	8	4	1	7
5(4M)	4	Liver extract	62	30	16	7	4	18	29
6(5M)	4	None	62	37	19	8	3	9	17
5(4M)	0	Liver extract	41	60	39	22	8	20	30
7(5M)	0	None	33	66	48	27	19	26	36

[1] Grams per 100 gm of milk solids.

[2] This refers to gain occurring only during the indicated period of time prior to the beginning of supplements.

[3] 200 μg per rat daily.

[4] Thiamine hydrochloride, 100 μg; riboflavin, 200 μg; pyridoxine, 100 μg; calcium pantothenate, 200 μg; nicotinic acid, 200 μg; and insositol, 400 μg per rat daily.

duplicate plates were made using eosin methylene blue agar for the coliform group and a special medium for the estimation of the total population. The latter medium (pH 7.2 to 7.4) contained yeast extract, 5 gm; glucose 5 gm; peptone, 5 gm; K_2HPO_4, 1 gm; liver infusion, 200 ml; and distilled water, 800 ml.

Rats which were not fed SST had approximately 3 to 12 million coliform organisms per gram of cecal contents whereas those given 5 or 10% SST had less than 100,000. The exact coliform count was not determined in many instances because 1–50,000 was the lowest dilution plated; in these cases such plates showed no coliform organisms. The total count on the controls not fed SST averaged over 800,000,000 per gm of cecal material. The bacterial count on the animals receiving 10% SST averaged about 400,000,000, and it was approximately 600,000,000 for the 5% SST group. Thus the total bacterial count was affected less than the coliform count. Nevertheless, the total number of bacteria decreased as the amount of SST was increased.

DISCUSSION

The growth data obtained by us do not agree with those of Welch and Wright ('44), and Wright, Skeggs, Welch, Sprague and Mattis ('45), who noted little if any growth impairment in rats fed mineralized dried milk (Klim) and SST. The reason for the difference is not apparent.

The findings indicate that much more of the sulfonamide is necessary under these conditions to effect marked growth impairment than when used in purified diets to which no p-aminobenzoic acid is added. It appears that this might be due, in part at least, to the effect of p-aminobenzoic acid which is present in considerable amounts in milk (Landy and Dicken, '42). This substance reduces the bacteriostatic action of SST (Day et al., '43). Since normally, "folic acid" may be synthesized in the intestinal tract, the presence of p-aminobenzoic acid would be expected to promote "folic acid" synthesis. Thus a larger concentration of SST would be required in milk

to produce growth impairment comparable to that in animals on p-aminobenzoic acid-low diets.

The slight prolongation in prothrombin time may be regarded as due to the combined effects of reduced vitamin K synthesis in the intestinal tract and a low concentration of this vitamin in evaporated milk. However, there is some evidence that the change may also be related to an effect of SST on the liver enzyme (s) responsible for prothrombin formation. Pilgrim and Elvehjem ('45) showed that feeding of SST to rats on purified diets resulted in sufficient hepatic injury to markedly reduce the activity of succinoxidase, malic oxidase and cytochrome oxidase.

SUMMARY

Weanling rats were restricted to mineralized evaporated and mineralized dried whole milk diets to which succinylsulfathiazole (SST) was added in amounts up to 10%.

Growth was markedly impaired at a level of 10% SST. The effect was less severe at lower concentrations. Either "folic acid" or solubilized liver concentrate caused prompt growth resumption. Xanthopterin alone, and a mixture of thiamine, riboflavin, pyridoxine, nicotinic acid, calcium pantothenate, and inositol, had no effect.

A slight prolongation in the prothrombin time occurred in animals given 10% SST. This returned promptly to normal when 2-methyl-1, 4-naphthoquinone was fed.

Marked reduction in the concentration of coliform bacteria in the cecum occurred in animals given SST. The total bacterial count was appreciably decreased.

The results are interpreted as further evidence of a low concentration of "folic acid" and vitamin K in evaporated and dried cows' milk.

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LITERATURE CITED

DAY, H. G., K. G. WAKIM, M. M. KRIDER AND E. E. O'BANION 1943 Effects of cecectomy, succinylsulfthiazole, and p-aminobenzoic acid on vitamin K synthesis in the intestinal tract of rats. J. Nutrition, vol. 26, p. 585.

DAY, H. G., K. G. WAKIM, W. H. ZIMMERMAN AND L. S. MCCLUNG 1944 Effects of succinylsulfathiazole on the intestinal flora and nutritional status of rats fed evaporated milk. J. Bact., vol. 48, p. 119.

KOSCHARA, W. 1943 Über Xanthopterin. Z. physiol. Chem., vol. 277, p. 159.

LANDY, M., AND D. M. DICKEN 1942 A microbiological method for the determination of p-aminobenzoic acid. J. Biol. Chem., vol. 146, p. 109.

MUELLER, W. S., AND A. W. WERTZ 1945 Vitamin K content of dairy and cacao products. J. Dairy Sci., vol. 28, p. 167.

PILGRIM, F. J., AND C. A. ELVEHJEM 1945 Enzymatic studies on tissues of rats fed purified rations containing succinylsulfathiazole. Arch. Biochem., vol. 6, p. 121.

SELLS, R. L., S. A. WALKER AND C. A. OWEN 1941 Vitamin K requirement of the newborn infant. Proc. Soc. Exp. Biol. Med., vol. 47, p. 441.

UNDERHILL, F. A., J. M. ORTEN, E. R. MUGRAGE AND R. C. LEWIS 1933 The effect of the prolonged feeding of a milk-iron-copper diet to rats. J. Biol. Chem., vol. 99, p. 469.

WELCH, A. D., AND L. D. WRIGHT 1944 The possible existence of a microbiologically inactive "folic acid"-like material possessing vitamin activity in the rat. Science, vol. 100, p. 153.

WILLIAMS, R. J., V. H. CHELDELIN AND H. K. MITCHELL 1942 The B vitamin content of milk from animals of different species. The University of Texas Publication, no. 4237, p. 97.

WRIGHT, L. D., H. R. SKEGGS, A. D. WELCH, K. L. SPRAGUE AND P. A. MATTIS 1945 The existence of a microbiologically inactive "folic acid"-like material possessing vitamin activity in the rat. J. Nutrition, vol. 29, p. 289.

APPARENT PROLONGATION OF THE LIFE SPAN OF RATS BY INTERMITTENT FASTING [1]

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ONE FIGURE

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INTRODUCTION

When a sufficient amount of choice food is available, laboratory rats, like many humans, eat enough to become more or less obese. As a consequence, the life span of rats feeding ad libitum, like the life span of their human counterparts, is presumably shortened. This inference is supported by the repeated findings of McCay and his associates ('42 a, b; '43) that the life span of rats can be considerably prolonged by a drastic restriction in their allowance of food. The findings of McCay and his associates practically constitute an experimental confirmation of the claims of Cornaro (Butler, '05) who attributed a considerable prolongation of his life to a rigid restriction of his food intake. However, since the time of Cornaro (1464–1566) no similarly prolonged and rigid voluntary restriction of the human food intake appears to have been recorded. Obviously, Cornaro's prolonged practice of food restriction has not been widely followed because a normal appetite tends to impel its more or less complete appeasement at reasonably frequent intervals, when sufficient palatable food is easily obtainable. Only short periods of food restriction, such as the religiously interdicted periods of food restriction or fasting of the past, would seem to be practical.

[1] This research was aided by a grant from Swift & Co., Chicago.

In the future however, a periodic practice of food restriction or fasting is likely to depend mainly on experimental evidence of its value. It therefore seemed of interest to determine whether periodic or intermittent fasting would serve to prolong the life span of rats or reduce or prevent the shortening of the life span which is presumably produced by feeding ad libitum.

A study was already made by Robertson, Marston and Walters ('34) of the effect of intermittent fasting on the life span of mice. In that study, twenty-four male and twenty-four female mice were fasted 2 successive days in 7. The average life span of the fasted males was found to be 745 days while that of twenty-four controls was 712 days. The average for the fasted females was 819 days while that of twenty-four control females was 773 days. However, the prolongation of life was not regarded as significant by Robertson and his associates. One criticism of their study is that littermate mice were not used as controls. Hence the individual life spans apparently varied too much to make the results seem significant. Another criticism is that no observations appear to have been made to determine whether the fasted mice remained free from peptic erosion or ulceration of the stomach and duodenum. This has been found to occur in some mice (and young rats) after single periods of starvation of 36 hours or more (Sun, '27; Hoelzel and Da Costa, '37).

The effect and after-effect of intermittent fasting on some aspects of growth and nutrition were also studied by von Seeland on chickens (1887), by Morgulis on salamanders ('13), by Kopec and Latyszwski on mice ('32) and by Kellermann on rats ('39) but the effect on the life span was not determined in any of these studies.

Observations previously made in this laboratory showed that rats fasted every other day and fed a diet low in protein between fasts developed peptic ulcers in the forestomach within about 2 weeks (Hoelzel and Da Costa, '32). However, rats fed a diet adequate in protein between single-day fasts usually remained free from peptic ulcers. With the use of a

diet relatively high in protein, no complication with peptic ulceration was therefore expected to develop in rats fasted 1 day in 3 or 4 but some doubts were still entertained whether rats could be fasted 1 day in 2 during prolonged periods without peptic lesions developing. In man, ulceration of the stomach is far less likely to occur while fasting because of an apparently lower fasting gastric acidity and the absence of the forestomach. Fasting 1 day in 2 or 3 by man also is apparently not the equivalent of fasting 1 day in 2 or 3 by the rat. However, in personal experiments, one of us (H.) found it impossible to maintain normal energy or remain free from nutritional edema while fasting every other day during periods of 2 to 5 months (Hoelzel, '43) but fasting 1 day in 3 immediately after having fasted 1 day in 2 during 5 months led to a recovery of energy and disappearance of nutritional edema. It was also found possible to recover fully from a 33-day fast in less than 33 days (Hoelzel, '44). Under these circumstances, it was deemed advisable to try various amounts of fasting in determining whether intermittent fasting would prolong the life span of rats.

In addition to various amounts of fasting, it also seemed advisable to try several diets. As a result, this study became somewhat complicated by the number of variables involved. The object of the present communication, however, is to report only the results of intermittent fasting on the life span of rats, independent of the specific effects of the different diets that were tried.

METHODS

In this study, 137 rats (60 males and 77 females), raised in the laboratory from rats obtained from The Wistar Institute, were used. These were all of the rats in seventeen litters with two or more of one sex or both sexes raised. The seventeen litters consisted of fourteen first litters and three second litters, with from two to thirteen raised rats in the individual litters. The rats were not weaned completely (separated from their mothers) until they were 35 days old.

Three omnivorous diets and one vegetarian diet were used. The omnivorous diets were a basic diet and two diets with 10% bulk-formers added to the basic diet. The basic diet consisted of 61.5% cooked and dried "whole veal," 31% corn starch, 2% powdered yeast, 1% cod liver oil, 1.5% inorganic salt mixture and 3% veal bonemeal. This diet provided approximately 35% proteins. The cooked and dried "whole veal" included practically all of the edible parts of calves, excepting excess fat and blood.[2] The first lot of this prepared veal contained 52% protein and 40% fat. Less fat was included in the preparation of subsequent lots but the composition of the original lot was approximated by adding fat when the diets were prepared. The second omnivorous diet consisted of the basic omnivorous diet plus 10% finely ground alfalfa stem meal. The third omnivorous diet consisted of the basic diet plus 5% psyllium seed husks and 5% specially prepared kapoc. The kapoc was mechanically cleaned, ground, boiled, washed, partly bleached, again washed and dried. The vegetarian diet consisted of 50% whole wheat flour, 10% peanut flour, 7% lima bean flour, 7% wheat gluten flour (containing 80% gluten), 7% corn gluten meal, 7% linseed meal, 5% powdered yeast, 5% alfalfa leaf meal and 2% NaCl. This diet provided approximately 30% proteins. Lettuce trimmings were supplied practically daily as a supplement to all of the diets. The control rats and the intermittently fasted rats while fed were kept continuously supplied with food.

Before the rats were 42 days old, all of them were supplied with the same food. This included some of each of the four experimental diets. When the rats became 42 days old, they were distributed so that some littermates of the same sex served in littermate tests of the effect of intermittent fasting or different amounts of intermittent fasting while other littermates served in tests of the effect of the different diets. Some rats with more than one littermate of the same sex consequently served as one of the littermates in 2 or more kinds of littermate tests. The intermittent fasting included fasting 1

[2] This was specially prepared for us by Swift and Co., Chicago.

day in 4, 1 day in 3 and 1 day in 2. The fasting was begun at the age of 42 days and was continued until the rats died.

RESULTS AND DISCUSSION

Effect of intermittent fasting on the life span

Figure 1 shows the distribution of the life spans of the individual control and fasted rats in 25-day periods. The life spans of rats with littermates only in the same group (but on different diets) are included. The average life spans of the

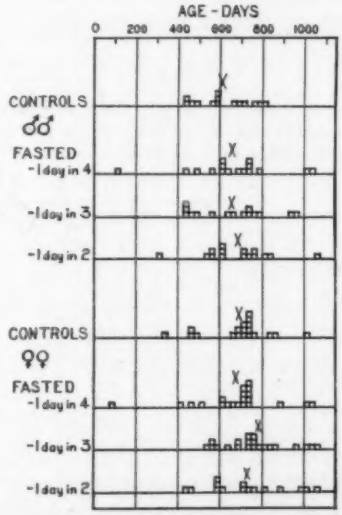

Fig. 1 Distribution of individual life spans of control and fasted rats in 25-day periods. Each square represents one rat. The average life span of each group is indicated by X. The life spans of rats with littermates only in the same group (but on different diets) are included.

groups are also indicated in figure 1 and in table 1. The results of the different amounts of fasting on littermates alone, with littermate controls fed identical diets ad libitum, are presented in table 2. Figure 1 and tables 1 and 2 show that, with the exception of the females fasted only 1 day in 4, the average life spans of all of the groups of fasted rats exceeded

that of the controls. Moreover, the data in table 2 indicate that the prolongation of life by fasting was practically proportional to the amount of fasting and that the life spans of the males were, on the average, increased more than the life spans of the females. However, this may merely mean that the life spans of the males were shortened more than the life spans of the females by feeding ad libitum.

The effect of fasting on littermate rats is also indicated by comparisons between the life spans of rats fasted 1 day in 4 or 3 and littermates fasted 1 day in 3 or 2, respectively. Data thus obtained and combined with the data in table 2 yield a

TABLE 1

Average life spans of control and fasted rats. These averages include the life spans of rats with littermates only in the same group but on different diets. Figures in parentheses indicate number of rats in each group.

SEX	AVERAGE LENGTH OF LIFE IN DAYS				
	Controls	Fasted			
		1 day in 4	1 day in 3	1 day in 2	All degrees
Males	612 (14)	658 (16)	653 (15)	683 (15)	664 (46)
Females	688 (19)	675 (21)	781 (22)	733 (15)	730 (58)

total of thirty male littermate comparisons and show that the average life span of the males was increased 90 days by fasting. Similarly obtained data on females show that the average life span in forty-five littermate comparisons was increased only 23 days by fasting. It seems noteworthy that, in spite of the substantial increase in the average life span of the males by fasting, the life span of the fasted males only approximated that of the control females (fig. 1 and tables 1 and 2).

A more detailed analysis of the results suggests that fasting 1 day in 4 and 1 day in 2 were complicated more by some extraneous factors than fasting 1 day in 3 or feeding ad libi-

tum. Thus, figure 1 shows that the earliest male and female deaths occurred in the groups fasted 1 day in 4 and the impression was that some of the other rats fasted 1 day in 4 also did not fare as well as most of the rats fasted 1 day in 3 or the controls. Perhaps the amount of food consumed in 3 days of feeding, with increased voracity but without proportionately increased capacity after 1 day of fasting, constituted a greater physiological overstrain than the amount of food consumed by the controls or by the rats fasted 1 day in 3. Figure 1 further shows that the males and females fasted 1 day in 2 also began dying earlier than the rats fasted 1 day in 3. Evidently fasting 1 day in 2 and beginning this at the age of 42 days was too much fasting for some rats. One of the females fasted 1 day in 2 apparently died of a hemorrhage from a chronic duodenal ulcer. Nothing like this was seen among over 2000 rats in a study of the production of peptic ulcers (Hoelzel and Da Costa, '37). More or less erosion and ulceration of the stomach was observed in other rats in this study but in most cases the lesions merely seemed to be due to premortal conditions, chiefly starvation due to loss of appetite associated with, or produced by, respiratory infections. (In about half of the rats with respiratory infections, no erosion or ulceration of the stomach occurs in spite of a complete loss of appetite or starvation and the usual postmortem changes in the intestines also are not seen. In such cases, all digestive secretions seem to be suppressed, excepting the secretion of a little bile.) Some erosion of the stomach may have occurred as a direct result of the experimental fasting among the rats fasting 1 day in 2 while they were still young. Moreover, female rats were previously found to develop more severe gastric lesions than males during prolonged starvation and a complication of this type may therefore explain why the females in this study did not benefit as much as the males from repeated single-day fasts. However, individual rats vary in their susceptibility to peptic erosion and ulceration and even some that were fasted 1 day in 2 evidently remained entirely free from such lesions. Both the male and the female

that lived longest among the 137 rats (1057 and 1073 days, respectively) were rats fasted 1 day in 2. The optimum amount of fasting for the average rat in this study nevertheless appears to have been fasting 1 day in 3 and the data in table 2 show that with this amount of fasting the life span of the males was increased about 20% and that of the females about 15%.

Effect of intermittent fasting on growth

Table 2 shows that the average weights of the intermittently fasted rats at 300 days were always lower than the average weights of their littermate controls but no drastic retardation of growth was produced by the fasting. In some cases, the average femoral lengths of the fasted rats at death were greater than, or equal to, those of the controls and, in other cases, the rats were only a little smaller. In short, intermittent fasting seems to make it possible to increase the life span to some extent without stunting the rats. Tests are in progress

TABLE 2

Showing the effect of different amounts of fasting on the weight, size (length of femur at death) and life span of littermate rats. Littermate controls fed identical diets ad libitum.

AMOUNT OF FASTING	NUMBER OF PAIRS OF LITTERMATES	AVERAGE WEIGHT (GM)				AVERAGE FEMORAL LENGTH AT DEATH (MM)		AVERAGE LENGTH OF LIFE (DAYS)		
		At 42 days		At 300 days						
		Controls	Fasted	Controls	Fasted	Controls	Fasted	Controls	Fasted	Difference due to fasting
Males										
1 day in 4	7	149	149	449	413	39.1	39.3	681	768	+ 87
1 day in 3	7	133	133	397	339	38.7	36.7	557	667	+ 110
1 day in 2	4	118	127	356	265	38.1	36.0	527	666	+ 139
All degrees	18	136	138	408	357	38.7	38.1	599	706	+ 107
Females										
1 day in 4	12	117	123	280	248	34.6	35.1	721	685	− 36
1 day in 3	10	118	120	291	258	34.8	35.2	708	814	+ 106
1 day in 2	5	113	114	276	234	34.5	34.1	614	768	+ 154
All degrees	27	117	120	823	249	34.7	35.0	696	748	+ 52

to determine whether the size of fasted rats can be more fully maintained and life can still be prolonged by beginning the fasting at 100 or 200 days, instead of at 42 days, and fasting the rats no more than 1 day in 3.

Are the results of fasting due, in whole or in part, to increased activity?

Tests made with rotary cages by Wald and Jackson ('44) revealed that rats run more when deprived of food, and McCay and his associates ('41) found that exercised rats lived longer than non-exercised rats. The activity of our fasting rats was largely limited to gnawing at the cages in attempts to escape. Many of the rats that were fasted 1 day in 4 or 3 seemed to become adapted to the fasting and remained at rest most of the time. The greatest unrest was manifested by some of the rats that were fasted 1 day in 2 but the life spans of the most restless rats were not the longest. The explanation seems to be that the greatest unrest or gnawing was manifested by the most voracious rats — the rats that apparently ate the greatest amounts of food between the days of fasting. The amount of food eaten may therefore have more than offset any possible benefit from increased exercise. In any event, the finding of McCay and his associates that rats subjected to forced exercise lived longer than non-exercised rats did not prove that the exercise per se increased the life span. The periods of forced exercise may merely have served to prevent the rats from eating as much as the controls in relation to their respective physiologic needs.

Influence of individual constitutions on the life spans

Constitutional similarities and differences among the individual rats, as determined by genetic factors and pre-experimental nutritional conditions, were obviously important factors determining the specific life spans. In the first place, a high degree of genetic uniformity in the Wistar strain evidently explains the death of 67% of the rats between the

ages of 550 and 850 days and the death of 85% between the ages of 400 and 900 days in spite of the use of four different diets and four different regimens of feeding or fasting. Some littermate rats, after having been kept from 400 to 1000 days on widely differing nutritional regimens, died within 24 hours or a few days of one another. Four of the twelve rats that lived to be over 1000 days old belonged to one of the seventeen litters. The view that rats in small litters are likely to be in a superior condition is supported by the finding that two males that composed one of the two smallest litters not only lived longer than eight (all) other males on similar nutritional regimens but also lived longer than fourteen (all) females on similar nutritional regimens. In contrast to this, the average life span of thirteen rats that composed one of the three largest litters was the lowest of any of the seventeen litters. However, independent of the size of the litters from which the rats came, the life span was found to be influenced more or less by the pre-experimental nutritional status or weight attained by the age of 42 days, when the rats were started on the specific experimental regimens. That is, the rats that were heaviest at the age of 42 days tended to live longest among the rats on the same regimen but this again was less true of the rats that were fasted 1 day in 3 or 2 than of the control rats or those that were fasted only 1 day in 4. That is, the prolongation of life due to fasting 1 day in 3 or 2 tended to outweigh the apparent handicap of a poorer nutritional start in life, as indicated by a lower than average pre-experimental weight. The data in table 2 show clearly that the heaviest group of male control rats lived longer than the lighter male controls and that their fasted littermates had correspondingly long life spans.

Influence of intermittent fasting on the development of disorders leading to death

Saxton ('45) showed that the development of inflammatory, neoplastic and degenerative diseases was delayed by the restriction of calories which increased the life span of the rats

in the experiments of McCay and his associates. Similarly, intermittent fasting seems to delay the development of the disorders which lead to death. Table 3 shows that a retardation of the development of mammary tumors, proportional to the amount of fasting, occurred in this study. These results support the observations previously made by Tannenbaum

TABLE 3

Development of mammary tumors in control and fasted rats

	CONTROLS	FASTED		
		1 day in 4	1 day in 3	1 day in 2
Number of females developing tumors	7	6	8	1
Per cent of females developing tumors	37%	29%	36%	7%
Earliest age at which a tumor began developing	437 days	458 days	675 days	775 days
Average age at which tumors began developing	628 days	613 days	783 days	..
Average life span of females with tumors	760 days	760 days	871 days	977 days
Weight of largest tumor	462 gm	220 gm	140 gm	26 gm
Average weight of tumors	193 gm [1]	67 gm [1]	36 gm	..
Average rate of growth of tumors (gm gained per 100 days)	134 gm [1]	48 gm [1]	42 gm	13 gm

[1] The weights of the tumors in 2 control rats and in 1 rat fasted 1 day in 4 were not recorded and the weight of a tumor in another rat fasted 1 day in 4 was excluded because the tumor was dehydrated. It became dehydrated because it was torn loose by the rat after it apparently began to interfere with defecation.

('40; '42) concerning the relation between the food intake and the development of tumors in mice. However, genetic factors may explain the occurrence of mammary tumors in all of the (six) females in one of the seventeen litters and the occurrence of ten of the other sixteen tumor cases in five pairs of littermates. The larger tumors became responsible for the

deaths of some of the females because of the tendency of the large tumors to ulcerate or become obstructive. The development of other types of tumors was apparently also retarded by intermittent fasting. The only tumor in the digestive tract, a fibrosarcoma in the forestomach, was found in the oldest control rat (a female 1012 days old). The tumor in the stomach was probably secondary to a tumorous condition of the uterus. Ulceration of the tumorous uterus evidently was responsible for the development of a fistula between the uterus and the ileum in this rat. Nothing comparable to this was seen among the fasted rats.

SUMMARY

Tests in which a group of thirty-three rats were allowed the same food ad libitum and groups of thirty-seven, thirty-seven and thirty rats were fasted 1 day in 4, 3 and 2, respectively, after the age of 42 days, showed that the apparent life span was increased by the intermittent fasting. The optimum amount of fasting appeared to be fasting 1 day in 3 and this increased the life span of littermate males about 20% and littermate females about 15%. However, the pre-experimental condition of the individual rats was also found to be an important factor determining the life spans.

No drastic retardation of growth was produced by the intermittent fasting but the development of mammary tumors was retarded in proportion to the amount of fasting.

LITERATURE CITED

BUTLER, W. F. 1905 The Art of Living Long. W. F. Butler, Milwaukee.

HOELZEL, F. 1943 Gastric acidity, nutritional hydration and appetite. Am. J. Digest. Dis., vol. 10, p. 121.

———— 1944 An explanation of appetite. Am. J. Digest. Dis., vol. 11, p. 71 and p. 101.

HOELZEL, F., AND E. DA COSTA 1932 Production of ulcers in the prostomach of rats by protein restriction. Proc. Soc. Exp. Biol. Med., vol. 29, p. 382.

———— 1937 Production of peptic ulcers in rats and mice by diets deficient in protein. Am. J. Digest. Dis. and Nutrition, vol. 4, p. 325.

KELLERMANN, J. H. 1939 The effect of intermittent starvation on calcification, food utilization and tissue composition. Onderstepoort J. Vet. Sci. and Animal Ind., vol. 12, p. 321.

KOPEC, S., AND M. LATYSZWSKI 1932 Untersuchungen über das Wachstum der Mäuse unter den Einfluss intermittierender Fütterung. II. Darreichung vollwertiger Nahrung, abwechselnd mit vollständiger Karenz. Biol. Generalis, vol. 8, p. 489.

MCCAY, C. M. 1942 a Nutrition, ageing and longevity. Trans. and Studies Coll. Physicians Philadelphia, 4 Ser., vol. 10, p. 1.

———— 1942 b Chemical aspects of ageing. Problems of Ageing, E. V. Cowdry, Ed., 2nd edition, p. 680. The Williams and Wilkins Co., Baltimore.

MCCAY, C. M., L. A. MAYNARD, G. SPERLING AND H. S. OSGOOD 1941 Nutritional requirements during the latter half of life. J. Nutrition, vol. 21, p. 45.

MCCAY, C. M., G. SPERLING AND L. L. BARNES 1943 Growth, ageing, chronic diseases, and life span in rats. Arch. Biochem., vol. 2, p. 469.

MORGULIS, S. 1913 The influence of protracted and intermittent fasting upon growth. Am. Naturalist, vol. 47, p. 477.

ROBERTSON, T. B., H. R. MARSTON AND J. W. WALTERS 1934 The influence of intermittent starvation and intermittent starvation plus nucleic acid on the growth and longevity of the white mouse. Aust. J. Exp. Biol. and Med. Sci., vol. 12, p. 33.

SAXTON, J. A., JR. 1945 Nutrition and growth and their influence on longevity in rats. Biological Symposia. Vol. XI. Jacques Cattell, Ed. Ageing and Degenerative Diseases. R. A. Moore, Ed., p. 177. The Jacques Cattell Press, Lancaster, Pa.

SUN, T. P. 1927 The effect of starvation and refeeding on the intestinal epithelium of the albino mouse. Chinese J. Physiol., vol. 1, p. 1.

TANNENBAUM, A. 1940 The initiation and growth of tumors. Introduction. I. Effects of underfeeding. Am. J. Cancer, vol. 38, p. 335.

———— 1942 The genesis and growth of tumors. II. Effects of caloric restriction per se. Cancer Research, vol. 2, p. 460.

VON SEELAND 1887 Ueber die Nachwirkung der Nahrungsentziehung auf die Ernährung. Biol. Centralbl., vol. 7, pp. 145, 184, 214, 246 and 271.

WALD, G., AND B. JACKSON 1944 Activity and nutritional deprivation. Proc. Natl. Acad. Sci., vol. 30, p. 255.

THE EFFECT OF DIFFERENT GRADES OF COCOA UPON THE RETENTION OF DIETARY CALCIUM BY GROWING RATS[1]

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Cocoa and chocolate are used extensively as flavoring materials in milk and ice cream, evidently in response to a consumer demand. Their use may be presumed to increase the consumption of these excellent calcium foods. The presence of 0.5 to 0.6% of oxalic acid in cocoa, however, suggests that cocoa, or chocolate, may impair the utilization of the calcium in milk and in ice cream by the formation of the very insoluble calcium oxalate, a form of calcium that is practically unavailable to the growing rat at least (Fairbanks and Mitchell, '38).

This possibility induced Mueller and Cooney ('43) to compare the retention of calcium by growing rats subsisting upon diets based upon whole milk powder with and without an addition of cocoa in the proportion of 0.84 part of the basal diet to 0.16 part of cocoa. The basal diet contained 0.554% of calcium and 0.472% of phosphorus. Twenty-one pairs of rats were used in addition to twenty-one litter-mate control rats killed and analyzed at the beginning of the experiment to permit an estimate of the initial content of calcium in the test rats. Within each pair of rats the consumption of the basal diet was equalized, while one of the rats received the cocoa powder in addition. After 5 weeks of feeding, the rats were sacrificed and analyzed for calcium and phosphorus.

[1] This investigation was aided by funds granted to the University of Illinois by the National Dairy Council on behalf of members of the chocolate industry.

The results of this well-conceived and well-executed experiment were clear-cut in their significance as revealed by statistical analysis. The rats fed the cocoa supplement, although receiving 19% more dry food, 6% more calcium and 30% more phosphorus than the rats on the basal diet only, grew only 89% as fast and deposited in their bodies only 76% as much calcium and 80% as much phosphorus. The effect of the cocoa supplement upon growth had been previously shown by Mueller ('42) to be traceable probably to its content of tannic substances. The primary effect upon mineral utilization was probably upon calcium, since (1) calcium was presumably a limiting factor in the diet, (2) calcium limitation will restrict phosphorus utilization, because the two minerals are so largely utilized together as a calcium phosphate in bone formation, and (3) the depression of phosphorus retention by cocoa was so nearly the same as that of calcium retention. However, the oxalic acid content of the cocoa could account for only about one-third of the observed depression in calcium retention. No clue to the residual causes of this depression was afforded by the data of the experiment or by the discussion of them by the authors. It should be noted that the ratio of milk solids to cocoa in the diets of this experiment was about 3.3 to 1, while in chocolate milk it is ordinarily about 13 to 1.

The cocoa that Mueller and Cooney used was a low-cost American process cocoa[2] containing 11.23% fat and 5.25% fiber. Cocoas of this general description are common at times on the market, being used in the manufacture of bakery goods and for low-cost chocolate syrups and confections, though there seems to be a considerable sale of such products for domestic use by certain retail stores throughout the country.

PLAN OF EXPERIMENTS

These experiments of Mueller and Cooney were repeated on growing rats at the University of Illinois, using essentially the same technic, but studying three different kinds of cocoa,

[2] Personal communication.

i.e., the low-cost brand used by the Massachusetts investigators and two medium-cost products, an American process breakfast cocoa and a Dutch process cocoa. The composition of these cocoas, in so far as they were analyzed in this laboratory, are summarized in table 1. In the Illinois experiments, the cocoas were incorporated into the basal diets at the expense of sugar, instead of being added to the diets, and all diets were equalized in fat content. The composition of the diets is given in table 2.

The effects of the different cocoas were studied in two experiments. In the first experiment, the effect of the two

TABLE 1

The chemical composition of the experimental cocoas.

DESCRIPTION OF COCOA	MOISTURE	ETHER EXTRACT	CRUDE PROTEIN	CRUDE FIBER	TANNIC SUBSTANCES [1]	THEOBROMINE	OXALIC ACID	ASH	CALCIUM	PHOSPHORUS
American process:	%	%	%	%	%	%	%	%	%	%
No. 1	4.70	22.45	22.62	3.90	10.57	1.79	0.504	4.97	0.121	0.668
No. 2 [2]	5.73	10.58	24.94	5.98	11.80	2.10	0.552	5.82	0.156	0.710
Dutch process:	5.20	23.33	21.12	3.61	6.62	1.44	0.512	8.50	0.124	0.640

[1] Corrected for ash.

[2] The same brand of cocoa used by Mueller and Cooney ('38).

medium-cost cocoas on the retention of the calcium and phosphorus of milk was studied with nine trios of rats, trio mates receiving equal amounts of their respective diets. In the second experiment, the effect of the low-cost cocoa was assessed with twelve pairs of rats, also receiving equal amounts of their respective diets. However, in both experiments the body weight gains of trio mates, or pair mates, were equalized by additions of sugar to the diet of the laggard rats, which were generally the rats receiving cocoa. This procedure was adopted because it could not be assumed that the net energy value of the cocoa was the same as that of the sugar it displaced in the cocoa diets. The experiments on individual pairs or trios were terminated after 4 to 9 weeks of feeding, de-

TABLE 2

The experimental diets.

CONSTITUENTS:	EXPERIMENT 1			EXPERIMENT 2	
	Diet 617	Diet 618	Diet 619	Diet 637	Diet 638
	%	%	%	%	%
Defatted dried beef	30	30	30	25	25
Non-fat dry milk solids (spray process)	19.47	19.47	19.47	19.47	19.47
Cocoa, American process no. 1	0	16	0	0	0
Cocoa, Dutch process	0	0	16	0	0
Cocoa, American process no. 2	0	0	0	0	16
Salt mixture Ca- and P-free	2	2	2	2	2
NaCl	1	1	1	1	1
$BaSO_4$	2	1.38	1.41	0	0
Wood Flock	0	0	0	2	1.04
Starch, vitaminized	5	5	5	5	5
Sucrose	28.62	16.83	16.94	16.80	10.12
Cerelose	0	0	0	16.80	10.13
Cod liver oil, fortified	1.5	1.5	1.5	1.5	1.5
Wheat germ oil	0.5	0.5	0.5	0.5	0.5
Lard	9.91	6.32	6.18	5	5
Mazola	0	0	0	4.93	3.24
Total	100.00	100.00	100.00	100.00	100.00
Calcium %					
Mix 1	0.225	0.244	0.242	0.268	0.292
Mix 2	0.261	0.275	0.277	0.272	0.292
Phosphorus %					
Mix 1	0.410	0.518	0.511	0.422	0.463
Mix 2	0.425	0.525	0.506	....	....
Protein %					
Mix 1	31.62	35.06	35.00	27.19	30.75
Mix 2	31.50	35.12	34.88	....	....
Calories per gm					
Mix 1	4.64	4.71	4.70	4.66	4.75
Mix 2	4.61	4.73	4.72	....	....

pending upon the rate of growth in each trio or pair, and the carcasses were analyzed for calcium and, in the first experiment, for phosphorus also.

The calcium and phosphorus in diets, cocoas and carcasses were determined by slight modifications of the methods of the Association of Official Agricultural Chemists. In the cocoa analysis, the tannic substances were determined by the method of Mueller and Kuzmeski ('44), the theobromine by the method of Wadsworth ('21), and the oxalic acid by a slight modification of the method of Majumdar and De ('38).

THE EXPERIMENTAL RESULTS

The average growth data in the two experiments are summarized in table 3. The differences in final weight and gain among trio or pair mates were largely due to differences in intestinal fill, since final weights relate to the empty carcasses. The differences in average attained body length among rats on comparable diets were insignificant statistically in the first ex-

TABLE 3

Average growth of rats on the experimental diets.

	EXPERIMENT 1			EXPERIMENT 2	
	Diet 617 No cocoa	Diet 618 American process cocoa no. 1	Diet 619 Dutch process cocoa	Diet 637 No cocoa	Diet 638 American process cocoa no. 2
Number of rats	9	9	9	12	12
Body weight —					
Initial, gm	47.0	46.0	47.2	47.2	47.4
Gain, gm	131.3	125.2	126.1	157.8	155.8
Body length, mm	198.7	199.6	199.9	206.1	203.6
Days on food	36	36	36	40	40
Total food eaten —					
Diet, gm	326.6	326.6	326.6	371	371
Sugar supplement —					
gm	3.8	24.4	20.1	0	70
pct. of diet	1.2	7.5	6.2	0	18.9

periment, but in the second experiment, the attained body length of the control rats receiving no cocoa averaged 2.5 mm greater than that of the rats receiving the low-cost cocoa, and this difference was significant with $P = 0.010$.

The amount of extra sugar required to maintain gains in the cocoa rats equal to those in the non-cocoa rats, in per cent of the basal food consumed, averaged 18.9 for the low-cost American process cocoa no. 2, 6.3 for the American process breakfast cocoa no. 1, and 5.0 for the Dutch process cocoa. The

TABLE 4

The average calcium metabolism data.

	EXPERIMENT 1			EXPERIMENT 2	
	Diet 617 No cocoa	Diet 618 American process cocoa no. 1	Diet 619 Dutch process cocoa	Diet 637 No cocoa	Diet 638 American process cocoa no. 2
Number of rats	9	9	9	12	12
Total calcium consumed, gm	0.763	0.821	0.817	1.002	1.084
Final calcium content, gm	1.203	1.130	1.111	1.453	1.273
Initial calcium content, gm	0.502	0.491	0.504	0.596	0.599
Calcium retained, gm	0.701	0.639	0.606	0.858	0.675

difference in this respect between the low-cost cocoa and the two medium-cost cocoas is highly significant, while the percentages for the two medium-cost cocoas were indistinguishable statistically.

The average calcium metabolism data are presented in table 4. Although receiving more dietary calcium than their littermate controls on cocoa-free diets, the cocoa rats stored considerably less calcium in their bodies in all trios and pairs. This situation is analyzed in detail for the individual rats in table 5, with statistical evaluations of average group differ-

TABLE 5

The effect of cocoa on the utilization of dietary calcium by growing rats.

PAIR OR TRIO NO.	AMERICAN PROCESS COCOA NO. 1				DUTCH PROCESS COCOA				AMERICAN PROCESS COCOA NO. 2			
	No-cocoa rats	Cocoa rats			No-cocoa rats	Cocoa rats			No-cocoa rats	Cocoa rats		
	Calcium retention in per cent of intake	Calcium retention in per cent of —			Calcium retention in per cent of intake	Calcium retention in per cent of —			Calcium retention in per cent of intake	Calcium retention in per cent of —		
		Total intake	Intake of non-cocoa calcium	Intake of non-oxalate calcium		Total intake	Intake of non-cocoa calcium	Intake of non-oxalate calcium		Total intake	Intake of non-cocoa calcium	Intake of non-oxalate calcium
1	95.2	74.6	80.5	86.3	95.2	77.6	83.3	88.6	88.3	68.3	73.9	77.5
2	99.3	88.9	95.5	101.9	99.3	89.3	95.6	100.0	91.8	68.8	74.4	78.1
3	94.6	74.6	80.3	85.8	94.6	68.5	73.4	78.1	88.2	61.4	66.4	69.6
4	96.9	78.5	84.3	89.9	96.9	61.7	66.0	70.1	79.3	68.1	73.7	77.3
5	84.8	84.8	91.8	98.7	84.8	85.0	91.3	97.4	92.5	64.1	69.3	72.7
6	72.9	67.1	71.9	76.6	72.9	73.6	78.6	83.4	85.6	65.1	70.9	74.8
7	95.0	76.2	81.7	87.1	95.0	61.7	66.1	70.2	79.2	55.5	60.0	63.0
8	100.0	77.1	83.0	88.9	100.0	76.0	81.4	86.6	84.1	64.2	69.4	72.8
9	88.1	83.2	90.1	96.9	88.1	81.8	87.9	93.7	81.5	57.3	61.9	65.0
10	..	..	..	..	..	..	..	..	87.9	54.3	58.8	61.7
11	..	..	..	..	..	..	..	..	85.8	57.5	62.2	65.3
12	..	..	..	..	..	..	..	..	85.8	59.8	64.7	67.9
Ave.	91.9	78.3	84.3	90.2	91.9	75.0	80.4	85.3	85.8	62.0	67.1	70.5
Mean deviation from control		13.6	7.6	1.7		16.9	11.5	6.6		23.8	18.7	15.3
S		8.35	8.69	9.09		13.70	14.28	14.77		5.65	5.97	6.19
t		4.58	2.43	.51		3.48	2.27	1.25		13.98	10.39	8.23
P		0.0009	0.018	0.31		0.0041	0.026	0.12		0.0001	0.0001	0.0001

ences. In this table, the calcium retentions for all rats are computed as percentages of the total calcium intakes, and for the cocoa rats, in percentages also, first, of the non-cocoa calcium in the diet, and, second, of the non-oxalate calcium, assuming that the oxalic acid of the cocoa combined with dietary calcium to its full capacity.

The average deviations of the percentage retentions of total calcium for the cocoa rats as compared with their controls were 23.8 for the rats receiving the low-cost American process cocoa no. 1, 13.6 for the rats receiving the medium-cost American process cocoa no. 2, and 16.9 for the medium-cost Dutch process cocoa, representing average depressions in total calcium retention of 27.7, 14.8 and 18.4%, respectively. Again, the detrimental effect of the low-cost cocoa exceeded statistically the effect of the two medium-cost cocoas. Each of the cocoas depressed significantly the utilization of the calcium in the basal diets, since the calcium retentions of the cocoa rats computed as percentages of the non-cocoa dietary calcium were significantly smaller than the percentage calcium retentions of trio-mate or pair-mate controls, but again the effect of the low-cost cocoa exceeded statistically those of the medium-cost cocoas.

When the calcium retentions of the cocoa rats are computed as percentages of the non-oxalate calcium in the diet, the values for the medium-cost cocoas still average less than the values for their controls, but the differences are insignificant, with probabilities that a random combination of uncontrolled factors could have produced as great or greater average differences than those observed, of 0.31 and 0.12. These probabilities are too large to be neglected and hence the differences may well have resulted from random factors only. It may be concluded, therefore, that the oxalic acid in these cocoas could have accounted for the total depression in calcium utilization brought about by their consumption. From the analyses of the cocoas given in table 1, it may be computed that these medium-cost cocoas contain enough calcium to combine with 54% of their contents of oxalic acid.

The low-cost American process cocoa no. 2 presented a different picture, however. In this case, the average retention of dietary calcium computed on the intake of calcium not bound by oxalic acid, is 70.5, as compared with a percentage utilization of 85.8 for the calcium in the non-cocoa diet, representing a difference that is highly significant with P=0.0001. The conclusion is clear, therefore, that with this cocoa the detrimental effect on calcium utilization is not due entirely to its content of oxalic acid, the effect of which is represented by the difference between 23.8, the depression in the percentage retention of total calcium, and 15.3, the depression in the percentage retention of non-oxalate calcium. Since this difference, 8.5, is about one-third of the total depression of 23.8, it appears that the oxalic acid in this cocoa accounts for only about one-third of its total depressing effect on calcium utilization. Thus, these results confirm completely those of Mueller and Cooney obtained with the same brand of cocoa.

The carcasses of the rats in the first experiment were analyzed for phosphorus as well as for calcium. Since the experimental diets contained about twice as much phosphorus as calcium (table 2), they were evidently not deficient in phosphorus. Nevertheless, the retention of phosphorus was definitely smaller in the rats receiving cocoa than in the control rats without cocoa. This depression in phosphorus was evidently occasioned by the smaller retention of calcium by the cocoa rats, since the two elements are to a large extent combined as a calcium phosphate in bone growth. For this reason, the ratios of retained calcium to retained phosphorus, with their standard errors, are nearly the same: 1.370 ± 0.037 for the non-cocoa rats, 1.314 ± 0.032 for the rats receiving the American process cocoa, and 1.303 ± 0.061 for the rats receiving the Dutch process cocoa. Using a "Student" ('25) analysis on paired differences, the probability that a random combination of uncontrolled experimental factors would have brought about average differences in ratio as large or larger than those observed is 0.08 for the comparison of control rats and the rats receiving American process cocoa, and 0.11 for

the comparison of control rats and the rats receiving Dutch process cocoa. These probabilities are too large to neglect, but small enough to suggest that these cocoas depressed calcium assimilation somewhat more than they depressed phosphorus assimilation. The small amount of phytin reported in cocoa (Arbenz, '22) does not seem to have affected phosphorus utilization.

CONCLUSIONS

1. Two medium-cost breakfast cocoas containing 22 to 24% of fat, one an American process and the other a Dutch process cocoa, depressed calcium assimilation in growing rats in proportion to their contents of oxalic acid. The cocoas themselves contained enough calcium to combine with 54% of their content of oxalic acid.

2. A low-cost American process cocoa containing 10.58% of fat depressed calcium assimilation to an extent about three times as great as could be accounted for by its content of oxalic acid.

3. The latter cocoa depressed the growth of rats also to a distinctly greater extent than the former two cocoas.

4. No distinction between the two medium-cost cocoas with reference to their effects upon growth or calcium metabolism was established.

LITERATURE CITED

ARBENZ, E. 1922 The phytin content of foods. Mitt. Lebensm. Hyg., vol. 13, p. 45.

FAIRBANKS, B. W., AND H. H. MITCHELL 1938 The availability of calcium in spinach, in skim milk powder, and in calcium oxalate. J. Nutrition, vol. 16, p. 79.

MAJUMDAR, B. N., AND N. K. DE 1938 The oxalic-acid content of some Indian foodstuffs. Indian J. Med. Res., vol. 25, p. 671.

MUELLER, W. S. 1942 The significance of tannic substances and theobromine in chocolate milk. J. Dairy Sci., vol. 25, p. 221.

MUELLER, W. S., AND M. R. COONEY 1943 The effect of cocoa upon the utilization of the calcium and phosphorus of milk. J. Dairy Sci., vol. 26, p. 951.

MUELLER, W. S., AND J. W. KUZMESKI 1944 The determination of tannic substances in commercial cocoa powders. J. Dairy Sci., vol. 27, p. 897.

"STUDENT" 1925 New tables for testing the significance of observations. Metron, vol. 5, p. 105.

WADSWORTH, R. V. 1921 The estimation of theobromine in cocoa and its products. Analyst, vol. 46, p. 32.